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OBSERVATIONS ON AMYLOLYTIC BACTERIA

I. A SURVEY OF NAMED MESOPHILIC SPECIES ON SOLUBLE STARCH[1]

BY EGON STARK[2] AND P. A. TETRAULT[3]

Abstract

A survey was made of the ability of known, named, mesophilic strains of 40 bacterial species to hydrolyze soluble starch in trypticase–yeast extract broth. The 40 species are either not described in Bergey's Manual as to their action on starch or they are listed as "not hydrolyzing starch". Strains of 35 species hydrolyzed soluble starch, including seven spp. described as nonamylolytic. The latter were: *Micrococcus pyogenes* var. *aureus*, *M. citreus*, *M. luteus*, *Escherichia coli*, *Erwinia carotovora*, *E. atroseptica*, and *Flavobacterium rhenanus*. Strains of four species failed to hydrolyze starch in repeated attempts: *Micrococcus sphaeroides*, *Gaffkya tetragena*, *Neisseria catarrhalis*, *Serratia rubida*. *Rhizobium trifolii* failed to grow. The disappearance of the substrate is a more permanent characteristic than either the accumulation of acids or of reducing sugars. Additional tests are required when the starch–iodine color is still blue after two weeks of incubation. The initial pH seems to influence amylase formation. Pertinent literature is quoted supporting the findings.

Introduction

The problem of calling a bacterial species amylolytic lies apparently in the difficulty of providing favorable cultural conditions for the formation of amylase and in the choice of criteria used to determine the action on starch. There exists a considerable amount of contradictory evidence in the literature concerning starch hydrolysis by bacteria. It is not uncommon to find the same species described as amylolytic by several independent investigators and as nonamylolytic by others. However, the formation of amylase by bacteria is a determinative characteristic of taxonomic importance. In Bergey's Manual of Determinative Bacteriology (4) slightly over 300 species are described as amylolytic, while the other descriptions (more than 1400) either do not include the action on starch or they state that the species "does not hydrolyze starch".

The aim of this investigation was to survey the starch hydrolyzing ability of strains of known, named, mesophilic, bacterial species, to study the reliability of criteria used for the detection of starch hydrolysis, and to investigate some of the factors necessary to obtain amylase formation. It was hoped to clarify the status of 40 species with regard to starch hydrolysis and

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to supplement some of the descriptions in Bergey's Manual on the basis of actual experimental findings and by quoting pertinent literature in support of these findings.

Materials and Methods

Forty-seven cultures, comprising 40 species, were tested. These cultures were part of the stock culture collection of the Department of Biological Sciences at Purdue University. They were chosen for this survey because the descriptions in Bergey's Manual either stated that the species "does not hydrolyze starch" or did not state the action on starch at all.

The medium consisted of trypticase (Baltimore Biological Laboratory) 1% and yeast extract (Difco) 0.5% in distilled water. To one-half of the medium 0.5% soluble starch (City Chemical Corp., N.Y.) was added. This starch was manufactured from potato starch. Each batch of medium was again divided into two parts which were adjusted to pH 6.0 and 7.0, respectively. The cultures were tested in duplicate on the following four treatments: pH 6.0 with and without starch, pH 7.0 with and without starch. Sterilization at 15 lb. pressure for 20 min. and subsequent incubation for two weeks did not destroy the property of the medium to give a typical blue iodine–starch color. Also, no reducing sugars were formed as evidenced by Fehling's test and a reaction of 6.8 was maintained without any adjustment.

Cells were scraped off the stock agar slant and inoculated into trypticase–yeast extract broth without starch. They were incubated for two days at their optimum temperature. One-tenth ml. inoculum (two drops) was transferred into 20 ml. of test medium contained in wide test tubes (25 × 150 mm.) and incubated for two weeks. At the end of the two weeks the cultures were tested with $N/10$ iodine solution (aqueous with potassium iodide) for the disappearance of starch, with a glass electrode for pH and with Fehling's solution for the formation of reducing sugars.

The iodine color test was performed as follows: one ml. of the inoculated medium was pipetted into a dry test tube. Two drops of the iodine solution were added. Also, tap water was added in sufficient amount to dilute the resulting color so that it could be recognized when held against transmitted light of a "daylight" fluorescent office lamp. When held against ordinary light bulbs different colors are produced. Too much dilution with tap water is undesirable and should be avoided. While it is sometimes difficult to tell which shade or hue of color exists, the difference between the uninoculated control and the fermented starch medium is unmistakable. The capital letters V (violet), P (purple), R (red), and C (colorless) in Table I denote starch hydrolysis, while b (blue) means that on the basis of the iodine test alone starch was not hydrolyzed. Brown and yellow were designated "colorless" in this report.

Fehling's test was either positive or negative for reducing sugars. The results are listed in Table I according to family and genus on the basis of Bergey's Manual. All species belonged to the order *Eubacteriales*.

TABLE I

THE ABILITY OF 40 SPECIES OF BACTERIA TO HYDROLYZE SOLUBLE STARCH

Species	Iodine color		Fehling's test		Without starch				With starch			
	Initial pH				Initial pH							
	6.0	7.0	6.0	7.0	6.0		7.0		6.0		7.0	
					Final pH							
Pseudomonadaceae												
Pseudomonas fluorescens	PP	PP	—	—	8.1	8.0	8.3	8.2	8.1	8.2	8.3	8.3
Pseudomonas syncyanea	RP	CR	—	—	8.0	8.0	8.1	8.1	8.0	8.0	8.3	8.1
Pseudomonas mildenbergii	PP	RR	—	—	6.2	7.8	8.1	8.0	7.8	7.8	8.1	8.0
Pseudomonas aeruginosa	CC	CC	—	—	8.5	8.5	8.6	8.6	8.6	8.6	8.5	8.6
Rhizobiaceae												
Rhizobium trifolii	*	*	*	*	*	*	*	*	*	*	*	*
Rhizobium japonicum	CC	CC	++	++	7.2	7.1	8.1	8.0	6.0	5.9	5.9	5.8
Rhizobium leguminosarum	*	CC	*	—	*	*	7.2	*	*	*	7.5	7.3
Micrococcaceae												
M. pyogenes var. *aureus* 6538	bb	Vb	—	—	8.7	8.7	8.8	8.9	8.7	8.7	8.3	8.9
M. pyogenes var. *aureus* 1047	PV	VV	—	—	7.9	7.9	8.1	8.1	7.4	7.5	7.6	7.7
Micrococcus citreus	bb	bb	—	—	8.2	8.2	8.2	8.3	6.6	6.4	6.4	6.4
Micrococcus epidermidis	VV	VV	−+	—	6.8	6.9	7.6	7.6	6.8	6.8	7.6	7.7
Micrococcus agilis	*	CC	*	−+	*	*	7.9	8.0	6.0	6.0	7.2	7.8
Micrococcus luteus	*	RR	*	—	*	*	7.9	7.9	6.0	6.0	8.0	8.0
Micrococcus lysodeikticus	bb	bb	—	—	6.0	5.9	7.7	7.7	6.0	5.9	5.9	5.8
Micrococcus sphaeroides	bb	bb	—	—	8.6	8.6	8.7	8.7	8.6	8.6	8.7	8.8
Gaffkya tetragena	bb	bb	—	—	7.5	7.3	7.5	7.6	7.1	7.1	7.5	7.5
Sarcina aurantiaca	PC	*	—	*	6.0	6.1	*	*	6.0	6.0	*	*
Sarcina aurantiaca 146	RR	RR	—	—	7.3	7.2	7.3	7.5	7.0	7.0	7.5	7.6
Sarcina flava	RV	RP	—	—	6.8	7.0	6.3	6.4	6.6	7.0	6.4	6.6
Sarcina ureae	VC	bb	—	—	6.6	6.6	8.0	8.0	6.6	6.7	8.0	8.0
Sarcina lutea	VC	CC	++	—	7.7	7.0	7.8	7.8	7.0	7.2	7.8	7.8
Neisseriaceae												
Neisseria catarrhalis	bb	bb	—	—	8.3	8.3	8.6	8.6	8.6	8.6	8.7	8.7
Achromobacteriaceae												
Alcaligenes faecalis	bV	bV	—	—	8.7	8.7	8.9	8.9	8.8	8.7	8.8	8.9
Alcaligenes viscosus	bb	VV	—	—	7.7	8.0	8.0	8.0	7.3	7.3	8.0	8.1
Flavobacterium aromaticum	CC	CC	++	+−	7.8	8.0	8.1	8.1	7.4	7.4	6.5	7.7
Flavobacterium devorans	bb	VV	—	—	7.5	7.5	7.8	7.9	6.7	6.8	6.1	7.3
Flavobacterium flavotenue	PP	PP	—	—	7.3	7.4	8.2	8.2	7.5	7.7	7.9	7.9
Flavobacterium lactis	PP	CC	—	—	7.7	7.8	7.9	8.0	6.5	6.8	7.5	7.6
Flavobacterium rhenanus	PP	PP	—	—	7.6	7.6	7.9	7.9	6.6	6.6	6.8	7.0
Flavobacterium rigense	PP	PP	—	—	6.9	7.2	7.7	7.8	6.7	6.7	7.1	7.4
Enterobacteriaceae												
Escherichia coli 4157	CR	RR	—	—	8.3	8.2	8.2	8.2	5.9	6.7	5.7	5.7
Escherichia coli 26	CP	RR	—	—	8.5	8.6	8.7	8.7	8.5	8.6	8.6	8.7
Escherichia coli B210	RV	RP	—	—	8.4	8.4	8.4	8.5	8.3	8.3	8.3	8.3

* *Failed to grow.*

NOTE: *b = blue; V = violet; P = purple; R = red; C = colorless.*

TABLE I—*Concluded*

THE ABILITY OF 40 SPECIES OF BACTERIA TO HYDROLYZE SOLUBLE STARCH—*Concluded*

Species	Iodine color		Fehling's test		Without starch				With starch			
	Initial pH				Initial pH							
	6.0	7.0	6.0	7.0	6.0		7.0		6.0		7.0	
					Final H							
Enterobacteriaceae—*Concluded*												
Klebsiella pneumoniae	Cb	CC	—	—	8.3	8.3	8.2	8.2	7.7	8.0	8.3	8.3
Erwinia carotovora	bb	VV	—	—	6.3	6.6	7.6	7.6	6.6	6.6	7.5	7.7
Erwinia atroseptica	VV	CC	—	++	6.7	6.9	7.9	7.9	7.1	7.1	6.3	6.5
Serratia marcescens 60	VV	VV	—	—	7.7	7.7	8.0	8.0	7.9	7.5	7.7	7.7
Serratia marcescens	RV	VV	—	—	7.9	7.9	8.2	8.3	7.9	8.0	7.6	7.7
Serratia kilensis	CC	CR	—	—	7.7	7.7	8.0	8.1	6.7	7.3	6.4	6.4
Serratia rubida	bb	bb	—	—	7.7	7.8	8.0	8.0	7.7	7.8	8.0	8.0
Proteus vulgaris	bb	bb	—	—	6.4	6.4	7.9	7.9	6.3	6.3	7.9	7.9
Salmonella enteritidis	CC	CC	−+	−+	8.4	8.6	8.4	8.5	8.3	8.3	8.3	8.3
Salmonella gallinarum	RP	PP	—	—	7.7	7.8	8.1	8.1	7.7	7.8	8.1	8.1
Salmonella pullorum	Vb	Vb	—	—	7.9	7.9	8.0	8.1	7.8	7.9	7.9	8.0
Salmonella schottmuelleri	RR	PP	—	—	8.4	8.4	8.5	8.5	8.2	8.2	7.9	7.9

NOTE: *b = blue; V = violet; P = purple; R = red; C = colorless.*

Results

Family II. PSEUDOMONADACEAE Winslow *et al.*

Genus I. *Pseudomonas* Migula

The following four species were tested at their optimum temperature of incubation: *P. fluorescens*, *P. syncyanea*, *P. mildenbergii* ATCC 795, all at 25° C., and *P. aeruginosa* Lederle 129 at 37° C.

On the basis of the iodine test, all four strains hydrolyzed soluble starch, regardless of the initial pH. No reducing sugars were present after two weeks of incubation. The final pH was alkaline in every instance.

P. aeruginosa was described as hydrolyzing starch by Crabill and Reed (8), Legroux and Genevray (29), and by Dowson (10). On the contrary, Stearn and Stearn (41) observed that *P. aeruginosa* did not attack starch at pH 5.5, 6.8, 7.3, and 8.4. Attempts by Legroux and Genevray (29) and by Vercellana (44) to obtain amylase from the interior of cells were unsuccessful.

P. fluorescens was described as amylolytic by Emmerling and Reiser (13), Crabill and Reed (8), Conn (7), Karstroem (24), Storck (42), and by Dowson (10).

There exist numerous descriptions of these two species and of the blue-green fluorescing members of this genus, generally, which mentioned that starch was not attacked: Clara (6), Seleen and Stark (39), Salvin and Lewis

(38). However, Lacey (28) noted that of 32 green fluorescing bacterial cultures, 11 were amylolytic, 16 were not, while 5 were not described as to their action on starch.

Family IV. RHIZOBIACEAE Conn

Genus I. *Rhizobium* Frank

R. trifolii ATCC 7023, *R. japonicum* 534-C, and a *R. leguminosarum* strain isolated from peas were tested at 25° C. *R. trifolii* failed to grow. *R. leguminosarum* grew only at the initial pH 7.0 and showed strong amylase activity. *R. japonicum* grew luxuriantly on all media and formed amylase very readily regardless of the initial pH. The presence of a large amount of reducing sugars was indicated by a strongly positive Fehling's test. The final pH was unchanged in the pH 6.0 cultures, but was acid when the initial pH was 7.0.

Raju (35) noted that 15 root nodule strains from different leguminous plants grew to some extent on soluble starch agar without changing the reaction of the medium. According to Fred, Baldwin, and McCoy (16), quoting Beijerinck (1888) and Mueller and Stapp (1925), root nodule bacteria are not amylolytic. McCoy (30) attempted to explain the utilization of starch during the formation of the root nodule without arriving at a satisfactory interpretation. Baldwin and Fred (3) tested 60 strains on 33 different fermentable substances, but did not include starch.

Family V. MICROCOCCACEAE Pribram

Genus I. *Micrococcus* Cohn

This genus has undergone a revision in the last edition of Bergey's Manual. Species belonging formerly to the genus *Staphylococcus* are now grouped in the genus *Micrococcus*.

M. pyogenes var. *aureus* ATCC 6538 (or FDA* 209P), *M. pyogenes* var. *aureus* Lilly 1047, *M. citreus*, and *M. epidermidis* were tested at 37° C., *Micrococcus agilis* and *M. luteus* ATCC 398 at 25° C., *M. lysodeikticus* ATCC 4698 at 30° C., and *M. sphaeroides* at 37° C. Bergey's Manual states that *M. pyogenes* var. *aureus* (formerly *Staphylococcus aureus*), *M. citreus*, and *M. luteus* "do not hydrolyze starch". The other species are not described as to their action on starch.

According to the iodine test, both strains of *M. pyogenes* var. *aureus* and one of *M. epidermidis* hydrolyzed soluble starch. At the completion of the experiment, no reducing sugars were present and all cultures had turned the medium alkaline. On the other hand, *M. citreus*, yielding a typical blue starch–iodine color, acidified the medium from pH 7.0 to 6.4. This effect was not noticeable when the initial pH was 6.0. In a repeated experiment, *M. citreus* yielded a purple iodine–starch color, while *M. epidermidis* gave a negative result.

* *Federal Drug Administration.*

On the basis of these observations, *M. pyogenes* var. *aureus*, *M. citreus*, and *M. epidermidis* hydrolyzed soluble starch.

M. agilis and *M. luteus* showed good amylolytic activity as evidenced by the iodine test and by the presence of reducing sugars in one test tube containing *M. agilis*. Both strains failed to grow in trypticase–yeast extract broth without starch when the initial pH was 6.0. *M. lysodeikticus* behaved like *M. citreus*: the iodine starch color was blue, but the pH of the medium was lowered from 7.0 to 5.8. *M. sphaeroides* was unable to hydrolyze soluble starch despite repeated attempts.

M. lysodeikticus is an amylolytic species, while *M. sphaeroides* is not.

No information could be found in the literature concerning the starch hydrolysis by *M. pyogenes* var. *aureus* because starch was not included among the fermentable substances. Vercellana (44) tried unsuccessfully to isolate amylase from the interior of cells of *Staphylococcus aureus*. Hucker (22) observed that of 29 strains of *M. citreus* only one formed acid from starch.

Genus II. *Gaffkya* Trevisan

G. tetragena "does not hydrolyze starch", according to Bergey's Manual. This was confirmed in repeated trials.

Fermi (14) and O'Neal (33) described this species as being amylolytic. O'Neal pointed out that only the R-type colonies hydrolyzed starch, while S-types did not.

Genus III. *Sarcina* Goodsir

S. aurantiaca, *S. aurantiaca* ATCC 146, and *S. flava*, tested at 30° C., *S. lutea* at 25° C., and *S. ureae* ATCC 6473 at 20° C., hydrolyzed soluble starch.

S. aurantiaca and *S. ureae* were only amylolytic when the initial pH of the medium was 6.0. *S. aurantiaca* failed to grow at pH 7.0; *S. ureae* grew but did not change the blue starch–iodine color. Whether *Sarcina* species require a slightly acid reaction for optimum amylase formation remains to be investigated in greater detail.

Reducing sugars were present in *S. lutea* cultures with an initial pH of 6.0. *S. flava* lowered the pH of the medium from 7.0 to 6.3 in trypticase–yeast extract broth with and without starch. This is additional evidence that pH changes are unreliable indicators of starch hydrolysis.

References in the literature concerning the starch-splitting ability of this genus are very scanty. Mees (31) found that dextrins, depending on their molecular size, were attached by beer sarcinae, but soluble starch was not hydrolyzed. Janke and Schaefer (23) observed that *S. erythromyxa*, not included in this survey, digested starch and that *S. lutea* was weakly amylolytic. Appleby (1) described an unidentified *Sarcina* sp. which digested starch after five weeks of incubation.

Family VI. NEISSERIACEAE Prevot

Genus I. *Neisseria* Trevisan

N. catarrhalis, tested at 37° C., failed to hydrolyze soluble starch.

Family IX. ACHROMOBACTERIACEAE Breed

Genus I. *Alcaligenes* Castellani and Chalmers

A. faecalis ATCC 212 and *A. viscosus* ATCC 337 were tested at 37° C. According to the iodine test, *A. faecalis* hydrolyzed soluble starch weakly, regardless of the initial pH of the medium. *A. viscosus* attacked the starch at pH 7.0, but not at pH 6.0. No reducing sugars were present at the end of the incubation period. All cultures turned the pH of the medium alkaline.

Genus III. *Flavobacterium* Bergey *et al.*

F. aromaticum, *F. rhenanus*, and *F. rigense* were incubated at 30° C., *F. devorans* and *F. lactis* at 25° C., while *F. flavotenue* was incubated at 20° C. According to Bergey's Manual, *F. rhenanus* "does not hydrolyze starch". The description of the other five species does not include their behavior on starch.

All species hydrolyzed soluble starch, as shown by the iodine test.

F. devorans did not change the blue starch–iodine color when the initial pH was 6.0. It showed slight activity at pH 7.0. One culture each of *F. devorans*, *F. aromaticum*, and *F. rhenanus* had an acid reaction when the initial pH was 7.0. All others turned the medium alkaline. Reducing sugars were only present in *F. aromaticum* cultures.

Darling and Sipple (9), studying soil bacteria from Antarctica, observed *F. devorans*, *F. flavescens*, and *F. solare* to be amylolytic. The last two species were not included in this survey, but are mentioned to show that more species of *Flavobacterium* are capable of starch hydrolysis than are listed in Bergey's Manual at present.

Family X. ENTEROBACTERIACEAE Rahn

Tribe I. ESCHERICHEAE Bergey, Breed, Murray

Genus I. *Escherichia* Bergey, Breed, Murray

Bergey's Manual describes *E. coli* as not hydrolyzing starch. In this survey, three different strains were tested: *E. coli* ATCC 26, *E. coli* ATCC 4157, and *E. coli* NRRL B210.

All strains were strongly amylolytic as evidenced by the iodine test.

E. coli ATCC 4157 lowered the pH from 7.0 to 5.7. All other reactions were alkaline. Reducing sugars were absent.

Numerous investigators reported that *E. coli* was capable of splitting starch: Fermi (15), Eijkman (12), Rosenthal and Patai (36), Crabill and Reed (8), Koch (25), Hill, Seidman, Stadnichenko, and Ellis (20), Karstroem (24), Stearn and Stearn (41), Dowson (10), Vaughn, Marsh, Stadtman, and Cantino (43).

Prange (34) quoted MacConkey (1908) and Kodama and Takeda (26) who observed weakly amylolytic *E. coli* strains. Prange studied 60 cultures on starch. Incubation was for 60 hr. He reported that the strains were nonamylolytic, although he mentioned that the indicator in the medium showed acid formation at first in many cultures.

O'Neal (33) observed that R-colonies of *E. coli* hydrolyzed starch while S-colonies did not. The width of the zone of clearing on starch agar plates was proportional to the degree of roughness: the rougher the colony the wider the zone.

Genus III. *Klebsiella* Trevisan

K. pneumoniae FDA 602 attacked starch vigorously. The final pH was alkaline and no reducing sugars were present. However, the iodine test was "colorless" indicating the disappearance of starch from the medium.

Fermi (15) listed *Bacterium pneumoniae* among starch splitting bacteria. Grimbert, quoted by Kruse (27), described pneumonia bacilli which attacked starch. It is difficult to tell whether these organisms were identical with *K. pneumoniae.* In this connection it may be mentioned that amylolytic pneumococci were reported by Salomon (37) and by Avery and Cullen (2).

Tribe II. ERWINEAE Winslow *et al.*

Genus I. *Erwinia* Winslow *et al.*

E. carotovora C3 and *E. atroseptica* E25 were tested at 25° C. Both cultures came originally from the collection of Dr. Burckholder.

E. atroseptica hydrolyzed soluble starch much better at pH 7.0 than at pH 6.0. Reducing sugars were present and the reaction was acid in cultures starting at pH 7.0.

E. carotovora hydrolyzed the starch when the initial pH was 7.0, but not at pH 6.0. The final reaction was alkaline; reducing sugars were not present.

Both species are described as "not hydrolyzing starch" in Bergey's Manual. However, on the basis of the above observations, *E. carotovora* and *E. atroseptica* are capable of hydrolyzing soluble starch.

Bacterium carotovorum was described as amylolytic by Dowson (10) and by Gorlenko and Voronkevich (19). On the contrary, Smith and Ramsey (40), testing six strains by the method of Eckford (11), did not observe starch hydrolysis.

Tribe III. SERRATEAE Bergey, Breed, Murray

Genus I. *Serratia* Bizio emend. Breed and Breed

S. marcescens ATCC 60 and *S. marcescens* (unnumbered) were incubated at 25° C., while *S. kilensis* and *S. rubida* were tested at 30° C. The red pigment formed by *S. marcescens* did not interfere with the iodine test.

Both strains of *S. marcescens* and the *S. kilensis* culture hydrolyzed soluble starch as shown by the iodine test. *S. kilensis* also lowered the pH of the starch broth from 7.0 to 6.4. No reducing sugars were present after two weeks of incubation.

S. rubida is a nonamylolytic species.

Eijkman (12) described *Bacillus ruber* as amylolytic. The identity of this organism is obscure, but it might have been a *S. marcescens* culture. Gorbach (18) quoted the work of Schneider (1894), Kraft (1902), Pappenhausen (1903), and Hefferan (1903) who observed that the addition of starch to the medium increased the pigment formation by *S. marcescens*. Pappenhausen noted that the pigmentation increased proportionally to the concentration of starch. This phenomenon does not necessarily mean that *S. marcescens* is amylolytic and evidence of this kind must be accepted with caution. It falls into the same category of evidence which states that better growth occurs in the presence of starch. More research is necessary to elucidate these relationships.

S. kilensis under the name *Bacillus ruber-balticus*, was described as amylolytic by Horowitz (21).

Tribe IV. PROTEAE Castellani and Chalmers

Genus I. *Proteus* Hauser

P. vulgaris is a nonamylolytic species. Wenner and Rettger (45) described 84 *Proteus* strains which did not hydrolyze starch.

Tribe V. SALMONELLEAE Bergey, Breed, Murray

Genus I. *Salmonella* Lignieres

S. enteritidis 149, *S. gallinarum* 469, *S. pullorum*, and *S. schottmuelleri* Squibb 12 were incubated at 37° C. On the basis of the iodine test, all four strains hydrolyzed soluble starch. The final pH was alkaline in every instance. Reducing sugars were only found in *S. enteritidis* cultures at pH 6.0 and 7.0. This species hydrolyzed starch according to Stearn and Stearn (41). *S. schottmuelleri*, under the name *Bacillus paratyphosus*, was described as amylolytic by Castellani (5).

The difficulty of calling a species amylolytic is perhaps well illustrated by the following observations on *Bacillus paratyphosus A*, now *S. paratyphi* and *B. paratyphosus B*, now *S. schottmuelleri:* Kodama and Takeda (26) and

Nobechi (32) described both organisms as nonamylolytic. Castellani (5) described both as amylolytic. Gildemeister and Herzberg (17), repeating the work of Kodama and Takeda, tested six strains of each species and found only one culture in each to be amylolytic.

Discussion

Soluble starch was hydrolyzed by strains of 35 of the 40 species surveyed. The four nonamylolytic species were: *Micrococcus sphaeroides*, *Gaffkya tetragena*, *Neisseria catarrhalis*, and *Serratia rubida*.

Of particular interest was the starch-hydrolyzing ability of strains belonging to species described in Bergey's Manual as "not hydrolyzing starch". These were: *Micrococcus pyogenes* var. *aureus*, *M. citreus*, *M. luteus*, *Escherichia coli*, *Erwinia carotovora*, *E. atroseptica*, and *Flavobacterium rhenanus*. Additional evidence of amylase formation by strains of these species was found in the literature. Consequently, the descriptions in Bergey's Manual ought to be emended accordingly.

Among the criteria used to detect amylase activity, the iodine color test seemed to be the best. In cases where the starch–iodine color is still blue after two weeks of incubation, it is suggested that the medium be tested for the accumulation of acids and of reducing sugars. If both are absent, the cultures should be incubated longer.

Testing daily for the accumulation of acids and of reducing sugars in the medium (without testing concomitantly for the disappearance of starch with iodine) constitutes a practice of doubtful value. After the starch molecule has been hydrolyzed by amylase, reducing sugars form: maltose directly from starch, glucose from maltose by the action of bacterial maltase. These sugars are only present in the medium if they have not been utilized by the bacteria. Therefore, positive sugar tests indicate starch hydrolysis, but negative tests are meaningless, unless more information is available.

The same reasoning holds true for the formation of acids: an acid pH indicates starch hydrolysis in most cases. It is unfortunate that many investigators have used this criterion exclusively when testing the bacterial action on starch. Since acid and sugar formation are only transient characteristics, they are not as reliable as the disappearance of the substrate.

The initial pH of the medium seemed to play a role in the formation of amylase as shown by the behavior of *Micrococcus pyogenes* var. *aureus* FDA 209 P, *M. luteus*, *M. agilis*, *Alcaligenes viscosus*, *Flavobacterium devorans*, *Erwinia carotovora*, *Sarcina ureae*, *S. aurantiaca*, and *Rhizobium leguminosarum*. However, more experimentation is required to elucidate this factor.

Little need be said about the strains which hydrolyzed starch actively in this survey. The cultural conditions were favorable for the formation of amylase. Of greater concern to the investigator are the weakly positive or negative cultures. Weak or no amylase formation may be caused by the type of soluble protein used. Work by the writers with thermophilic bacteria,

grown on eight different commercial protein media, has shown that no single medium supported the formation of amylase by all 14 strains tested. By changing the protein source of the medium it was possible to obtain starch hydrolysis by apparently negative cultures.

A species should never be referred to as "weakly" or "strongly" amylolytic. Strains of a species do or do not produce amylase under a certain set of conditions. Only the action on starch can be described as weak or strong on the basis of the iodine test. Weak amylolytic activity means two things: (1) the organism forms amylase; (2) the cultural conditions are unfavorable for optimum formation of amylase. It is possible to obtain maximum production of amylase by carefully manipulating the medium and the cultural conditions. The important thing from a taxonomic point of view is the presence or the absence of a certain enzyme which might be used to describe and/or separate species. The descriptive terms "weak" or "strong" with regard to enzyme formation have little meaning in determinative work.

An organism once reported to be amylolytic should be considered as such despite evidence to the contrary. It is more difficult to demonstrate the presence of amylase than its absence. Negative findings in cases of known amylolytic species are merely an indication of inadequate environmental conditions. Strains of the organism, colony morphology, type of protein, type of starch, other constituents of the medium, initial pH, length of incubation, and criteria used to detect amylase activity are some of the factors that must be closely observed in studies of this kind.

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OBSERVATIONS ON AMYLOLYTIC BACTERIA

II. A SURVEY OF NAMED MESOPHILIC SPECIES ON FIVE DIFFERENT STARCHES[1]

By Egon Stark[2] and P. A. Tetrault[3]

Abstract

The ability of strains of 40 mesophilic species of bacteria to hydrolyze five different starches was studied. These species are either not described in Bergey's Manual as to their action on starch or they are listed as "not hydrolyzing starch". Strains of 32 species were amylolytic, six species were not. Strains of two species failed to grow. Of the amylolytic species, 22 attacked all five starches, two attacked four, six hydrolyzed two, and two species hydrolyzed only one starch. No single starch was hydrolyzed by all strains of 32 amylolytic species. Rice and soluble starch were most readily hydrolyzed. Potato and corn starch offered the greatest resistance at first. Arrowroot starch held an intermediate position. Two weeks of incubation seemed to be a minimum period. The longer the cultures were incubated the more completely was a particular starch hydrolyzed and the more numerous were the amylolytic cultures. The implications of these observations for determinative work are discussed.

Introduction

A preliminary survey of the ability of strains of 40 known, named, mesophilic species of bacteria to hydrolyze soluble starch revealed that 35 species were amylolytic (7). Since the type of starch used is one of the factors influencing amylase formation by microorganisms, the survey was extended to include five different starches. Soluble starch was one of the starches in order to be compared with the nonsoluble starches and to serve as a replication of the first experiment.

The aims of the extended survey were to obtain additional evidence of the starch hydrolyzing ability of the strains used, to study the influence of different starches on the formation of amylases by bacteria, and to determine the effect of the length of the incubation period.

Materials and Methods

A large batch of trypticase (BBL)* 1%–yeast extract (Difco) 0.5% broth was prepared, adjusted to pH 7.0, and subdivided into five portions. A slurry of the respective starch was added to each batch to give a final concentration of 0.5%. The media were tubed in wide test tubes (25 × 150 mm.) and sterilized at 15 lb. pressure for 20 min. The following starches

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were used: soluble starch* (City Chemical Corp., N.Y.), corn starch (General Chemical Co., N.Y.), rice starch (Coleman and Bell Co., Norwood, Ohio), and potato and arrowroot starch (Baker Chemical Co., Phillipsburg, N.J.).

The inoculum consisted of 0.1 ml. (two drops) of a 24-hr.-old culture grown in trypticase–yeast extract broth without starch. The media were inoculated in duplicate sets and were incubated at the optimum temperature of each species.

The 45 strains, belonging to 40 species, were the same as were used in the preliminary survey.

The hydrolysis of starch was determined by testing the media with $N/10$ iodine solution (aqueous with potassium iodide) on the fourth, eighth, and 14th day of incubation. The exact procedure, interpretation, and abbreviations employed are the same as described in the first survey.

Results are shown in Tables I and II.

Results

Strains of 32 species hydrolyzed one or more starches, six were not amylolytic, and two failed to grow. Amylase activity by the positive cultures was not uniform on all starches.

Strains of the following species hydrolyzed all five starches: *Pseudomonas fluorescens, P. syncyanea, P. mildenbergii* ATCC 95, *P. aeruginosa* Lederle 129; *Rhizobium japonicum* 534-C; *Micrococcus agilis, M. luteus* ATCC 398; *Sarcina aurantiaca* (*S. aurantiaca* ATCC 146 did not hydrolyze potato and corn starch), *S. lutea; Alcaligenes viscosus* ATCC 337; *Flavobacterium aromaticum, F. lactis, F. devorans, F. flavotenue, F. rhenanus, F. rigense; Escherichia coli* ATCC 26, ATCC 4157, and NRRL B210; *Klebsiella pneumoniae* FDA 602; *Serratia marcescens* (*S. marcescens* ATCC 60 did not hydrolyze soluble starch), *S. kilensis; Salmonella enteritidis* and *S. schottmuelleri* Squibb 12.

Cultures of the following species hydrolyzed four starches: *Alcaligenes faecalis* ATCC 212 (not soluble starch), *Erwinia atroseptica* (not rice starch).

Strains of the following species attacked only two starches: *Micrococcus pyogenes* var. *aureus* Lilly 1047 (hydrolyzed rice and soluble starch). *M. pyogenes* var. *aureus* ATCC 6538 attacked only soluble starch. M. *citreus* attacked rice and soluble starch. *M. lysodeikticus* ATCC 4698 hydrolyzed rice and potato starch. *Sarcina flava* acted on rice and arrowroot starch. *Erwinia carotovora* C3 hydrolyzed rice and soluble starch, while *Salmonella gallinarum* attacked only rice and soluble starch.

One starch only was hydrolyzed by the following cultures: *Micrococcus epidermidis* (rice starch) and *Salmonella pullorum* (soluble starch).

The nonamylolytic species included: *Micrococcus sphaeroides, Serratia rubida, Proteus vulgaris, Gaffkya tetragena, Neisseria catarrhalis,* and *Sarcina ureae.*

* *Manufactured from potato starch.*

Two strains failed to grow: *Rhizobium trifolii* ATCC 7023 and *Rhizobium leguminosarum* from peas.

Contrary to the results obtained in the preliminary survey (7) *Micrococcus citreus* gave a purple iodine reaction on soluble starch, while *M. epidermidis* did not change the iodine color. *Alcaligenes faecalis* hydrolyzed all starches except soluble starch, which was hydrolyzed in the first experiment. The same was true of *Serratia marcescens* ATCC 60. These inconsistencies reflect the complex nature of the substrate and the difficulty of obtaining identical results in repeated experiments.

Bergey's Manual (1) lists the following species as "not hydrolyzing starch": *M. pyogenes* var. *aureus*, *M. citreus*, *M. luteus*, *Escherichia coli*, *Erwinia carotovora*, *E. atroseptica*, and *Flavobacterium rhenanus*. The data presented in Table I give evidence of the ability of strains of these organisms to hydrolyze various starches. Their ability to attack soluble starch was demonstrated in the preliminary survey. The weak activity exhibited by the two strains of *Micrococcus pyogenes* var. *aureus* and by *M. epidermidis* might be an indication that the cultural conditions were not favorable for optimum amylase formation. Additional research is necessary to clarify this feature. The same is true for *Salmonella pullorum*.

The species which failed to hydrolyze any of the five starches, with the exception of *Sarcina ureae*, which needs an initial pH of 6.0 to form amylase, must be considered to be nonamylolytic until evidence to the contrary is forthcoming.

The Comparative Usefulness of Different Starches for the Formation of Amylase by Bacteria

The 32 amylolytic species did not hydrolyze all starches uniformly. It appeared that certain starches were more susceptible to hydrolysis than others. This raises the question of the comparative usefulness of various starches for the demonstration of amylase formation by bacteria. Table II shows the number of cultures which hydrolyzed various starches over a period of two weeks.

Thirty-seven of the 45 strains tested were amylolytic and constitute 100% in the above tabulation.

The results obtained after four days of incubation reflect the ease with which a certain starch was hydrolyzed. Rice starch was most readily attacked, the number of amylolytic cultures being twice as large as on potato starch. After two weeks of incubation this difference had narrowed down considerably.

The length of the incubation period is another factor of major importance in determining the total number of amylolytic cultures. The longer the cultures were incubated the more thoroughly was the particular starch attacked and the larger was the number of amylolytic cultures. Two weeks of incubation seemed to be a minimum period for the hydrolysis of starch under the above conditions.

TABLE I

THE STARCH-IODINE COLOR OF 40 SPECIES ON FIVE DIFFERENT STARCHES

Species	Starch														
	Soluble			Potato			Corn			Rice			Arrowroot		
	Days incubated														
	4	8	14	4	8	14	4	8	14	4	8	14	4	8	14
P. fluorescens	bb	VV	PV	bb	VV	VV	VV	VV	VV	VV	PV	CV	bV	PV	CV
P. syncyanea	bb	PP	RP	bb	VV	VV	bb	PV	CV	bP	CP	CP	bb	PP	PP
P. mildenbergii	bb	VV	VV	bb	VV	VV	bb	bP	bP	bV	VV	VV	VV	VV	VV
P. aeruginosa	VP	VP	VP	bP	VR	CV	VV	VV	VV	VV	VV	VV	PP	PP	CP
*R. japonicum**	CC	—	—	CC	—	—	CC	—	—	CC	—	—	CC	—	—
M. pyogenes var. *aur.* 6538	VV	VV	VP	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
M. pyogenes var. *aur.* 1047	bV	bV	bV	bb	bb	bb	bb	bb	bb	bb	bV	bV	bb	bb	bb
M. citreus	bb	bb	VP	bb	bb	bb	bb	bb	bb	bb	bb	bV	bb	bb	bb
M. epidermidis	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bV	bb	bb	bb
M. agilis	bb	PP	PC	bb	bV	CC	bb	bR	CC	bb	bV	CC	bb	VV	RR
M. luteus	bb	bV	RR	bb	bb	RR	bb	bV	CC	bb	VR	CC	bb	VV	RR
M. lysodeikticus	bb	bb	bb	bb	bP	bP	bb	bb	bb	bb	bb	VV	bb	bb	bb
M. sphaeroides	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
G. tetragena	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
S. aurantiaca	bb	RR	RR	bb	bb	VP	bb	PP	PC	bb	PC	CC	bb	VR	CC
S. aurantiaca 146	bb	PP	RR	bb	bb	bb	bb	bb	bb	bb	VV	VV	bb	bb	bb
S. flava	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	VV	VV	bb	bC	VC
S. lutea	bb	RR	CC	bb	bb	CR	bb	VR	PC	bb	bV	bC	bb	bP	CC
S. ureae	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
E. coli ATCC 26	RV	RR	RC	VV	VV	VV	VV	VV	VP	VV	VV	VV	VV	VV	VV
E. coli ATCC 4157	RP	CC	—	bV	PR	CC	VV	PP	CC	VV	VC	CC	RR	RC	CC
E. coli NRRL B210	VV	PP	PP	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VV	VR
K. pneumoniae	VV	VV	CC	bC	bC	CC	CC	—	—	CC	—	—	bb	CC	—

* *Rhizobium trifolii ATCC 7023 and Rhizobium leguminosarum failed to grow.*

NOTE: *b = blue; V = violet; P = purple; R = red; C = colorless.*

TABLE I—*Concluded*

THE STARCH-IODINE COLOR OF 40 SPECIES ON FIVE DIFFERENT STARCHES—*Concluded*

Species	Starch														
	Soluble			Potato			Corn			Rice			Arrowroot		
	Days incubated														
	4	8	14	4	8	14	4	8	14	4	8	14	4	8	14
A. faecalis	bb	bb	bb	bV	bV	bV	bb	bb	VV	bb	VV	PP	bb	bb	VV
A. viscosus	bb	bP	VP	bb	bb	PV	bb	bb	VV	bb	VV	PP	bb	VV	VV
E. carotovora	bb	bV	VV	bb	bb	bb	bb	bb	bb	bb	VP	VP	bb	bb	bb
E. atroseptica	bb	bV	VP	bb	bb	bb	bb	bb	VV	bb	bb	bb	bb	VP	PR
P. vulgaris	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
S. marcescens	PV	PR	CC	VV	VV	VP	VV	VV	VV	VV	VV	VV	VV	VV	VV
S. marcescens 60	bb	bb	bb	bb	bb	PP	bb	VV	CC	bb	VV	VV	bb	bV	VV
S. kilensis	bb	bP	PV	bb	bV	VV	bb	bV	bV	bV	VV	VV	bb	VR	VC
S. rubida	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
F. aromaticum	bV	CC	—	bV	CC	—	bC	CC	—	CC	—	—	VV	VC	VC
F. devorans	bb	VV	VV	bb	bb	VV	bb	bb	VC	bb	bV	bP	bb	VV	PP
F. flavotenue	bb	VV	VV	bb	bb	VV	bb	bb	VV	bb	bV	VP	bb	bV	VV
F. lactis	bb	VV	VV	bb	bb	VV	bb	VV	VR	VP	VP	VP	bb	VV	VV
F. rhenanus	bb	VP	VP	bb	bb	VV	bb	VV	VV	bP	PP	PC	bb	bV	VV
F. rigense	bb	VV	PP	bb	bb	VV	bb	bb	VV	bb	VV	VP	bb	bb	VV
N. catarrhalis	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
S. enteritidis	PP	CC	—	bb	VP	CC	VV	VP	VC	bV	bV	bV	VV	VR	VC
S. gallinarum	bb	bb	VP	bb	bb	bb	bb	bb	bb	bV	bV	bV	bb	bb	bb
S. pullorum	bb	bb	bV	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb	bb
S. schottmuelleri	VV	VP	PP	bb	bV	VP	bb	VV	VV	PV	PV	PV	bP	bP	bP

* *Rhizobium trifolii ATCC 7023 and Rhizobium leguminosarum failed to grow.*

NOTE: *b = blue; V = violet; P = purple; R = red; C = colorless.*

TABLE II

THE EFFECT OF THE TYPE OF STARCH AND OF THE LENGTH OF THE INCUBATION PERIOD ON THE AMYLASE ACTIVITY OF MESOPHILIC BACTERIA

Starch	Amylolytic cultures						False negative results	
	Days						Days	
	4	8	14	4	8	14	14	
	Numbers			Per cent			Number	Per cent
Rice	17	31	34	45.9	83.7	91.9	3	8.10
Soluble	12	29	32	32.4	78.4	86.5	5	13.5
Arrowroot	11	26	28	29.7	70.3	75.7	9	24.3
Corn	10	21	27	27.0	57.7	73.0	10	27.0
Potato	9	16	27	24.3	43.2	73.0	10	27.0

In Table II the heading "false negative results" refers to those amylolytic cultures which failed to attack the particular starch. The three strains which did not hydrolyze rice starch attacked some other starch and are, therefore, amylolytic. The magnitude of error introduced by these false negative results ranged from 8 to 27%. This error is too large to be ignored. It is, perhaps, the reason why many species have been reported in the past as being unable to hydrolyze starch.

Discussion

Several investigators have experimented with different starches for various reasons.

Wortmann (9), among the first investigators to put amylase studies with bacteria on an experimental basis, used bean, canna, curcuma, iris, palm, potato, and wheat starch. He noted that wheat grains were most readily attacked by bacteria. All starches, except potato starch, showed signs of bacterial corrosion after eight days of incubation.

Duclaux (4) studying the formation of alcohol by *Amylobacter butylicus* and *Amylobacter ethylicus*, two organisms which he had isolated from potatoes, observed that rice, tapioca, and wheat starch gave varying amounts of alcohol. He ascribed the difference in yield to the origin of the starch.

Eijkman (5) mentioned briefly in a survey of amylolytic bacteria that for starch agar plates rice or arrowroot starch was better suited than potato starch.

Yet, despite these early observations on the usefulness of starches other than potato starch, the latter remained the favorite substrate for amylase studies. Availability might have been responsible for this.

Laybourne (6) tried to distinguish between fecal and nonfecal strains of *Aerobacter aerogenes* by using different starches. The following 15 starches were used: arrowroot, barley, bean, buckwheat, canna, corn, ginger, lentil,

* *Rhizobium trifolii ATCC 7023 and Rhizobium leguminosarum failed to grow.*
NOTE: *b = blue; V = violet; P = purple; R = red; C = colorless.*

oat, pea, potato, rice, sago, tapioca, and wheat. The medium consisted of 0.5% starch, 1% peptone (Witte), and 0.5% dipotassium phosphate in distilled water. Methyl red was used to indicate acid formation and Durham fermentation vials to indicate gas. The cultures were incubated at 37° C. for one week and then tested with Fehling's and Barfoed's solutions for the formation of sugars. All sugar tests were negative. Gas was present in 46 of the 49 strains on all starches and in the other three cultures on most starches. The final pH was extremely variable; each starch yielding acid, neutral, and alkaline reactions. Laybourne concluded that on the basis of the results obtained no distinction could be made between fecal and nonfecal strains of *Aerobacter aerogenes*.

Castellani (2) used the same 15 starches, but replaced buckwheat by yam starch. He wanted to distinguish between the following groups of pathogenic bacteria: (*a*) *Bacillus paratyphosus* A and B and C, (*b*) *B. dysenteriae* Shiga and Flexner and Y of Hiss and Russel and Duval, (*c*) *B. ceylonensis* A and B. The medium was that of Laybourne. Readings for gas and acidity were taken after 7, 14, and 21 days of incubation. The results were sufficiently clear-cut to enable Castellani to distinguish between the different strains. He was also able to identify different starches by using one organism.

Crabill and Reed (3) recommended the use of corn starch for starch agar plates. However, their results with bacteria did not support their recommendation. Vaughn and Levine (8) also used corn starch "since it is important that the starch used be free from reducing substances . . . soluble starches are unsatisfactory for differential purposes as most coliform bacteria readily ferment them, since they contain products of starch hydrolysis".

Soluble starch behaved typically like starch in both surveys. After sterilization and incubation for two weeks at temperatures as high as 65° C. (in work with thermophiles), soluble starch in uninoculated control tubes gave a typical deep blue color when tested with iodine, showed no presence of reducing sugars when tested with Fehling's solution, and maintained a reaction of 6.8 without any adjustment. The fact that several cultures were unable to hydrolyze it is evidence that soluble starch is just as suitable for amylase investigations as nonsoluble starches. On the contrary, it is easier to manipulate when in solution and does not form gels which resist hydrolysis.

On the basis of the results obtained in both surveys it is recommended to use soluble and rice starch concomitantly in determinative work since both starches are readily hydrolyzed by bacteria capable of producing amylase. It may be noted from Table I that the following cultures were nonamylolytic on soluble starch, but amylolytic on rice starch: *Micrococcus epidermidis* (doubtful positive), *M. lysodeikticus*, *Sarcina flava*, *Alcaligenes faecalis*, and *Serratia marcescens* 60. The following cultures were amylolytic on soluble starch, but nonamylolytic on rice starch: *M. pyogenes* var. *aureus* 6538, *Erwinia atroseptica*, and *Salmonella pullorum* (doubtful positive). These two

starches brought out the amylolytic properties of all the positive cultures. Although some cultures gave negative results with one starch they hydrolyzed the other.

There exist marked differences between brands of the same type of starch and even between batches of the same starch. This is being investigated at present.

The investigator who is engaged in determinative work and who is testing large numbers of cultures under uniform conditions for starch hydrolysis is interested in eliminating as many "false" negative results as possible. By false results is meant the apparent inability of certain strains to attack starch, although they are known to form amylase. Since the mechanism of amylase formation is not known, testing for amylase activity at the present time is largely a hit-and-miss proposition. For example, it is not known whether the enzyme is secreted by the living cell or whether it is released into the medium after lysis of the cell. Also, relatively little is known concerning the cultural conditions which may enhance or inhibit amylase activity. The constituents of the medium, especially certain buffer salts, seem to regulate amylase formation. The following factors definitely influenced amylase activity in both surveys: type of starch used, length of incubation, initial pH of the medium, strain used, and the methods applied to detect amylase activity.

Some starches resisted hydrolysis more than others. Potato and corn starch, in particular, were not hydrolyzed by many cultures (27%) although they acted rather strongly on other starches.

Prolonging the length of the incubation time resulted in larger numbers of amylolytic cultures and in a more thorough hydrolysis of the starch. Two weeks appeared to be a minimum period for amylase studies with mesophiles. It is therefore recommended that cultures be incubated for at least two weeks. Initial reaction of the medium influenced the amylase formation by some cultures, but not by others. The optimum pH for amylase formation by each culture should be determined experimentally before testing for starch hydrolysis.

Strain differences have been observed and must be guarded against.

Testing with iodine for the disappearance of the substrate was found to be a more reliable criterion of amylase activity than testing either for the accumulation of acids or of reducing sugars. Amylase formation is indicated when the iodine color is other than blue, when acids are present and when reducing sugars can be demonstrated. In case of negative findings it is necessary to incubate the cultures longer or to apply all tests simultaneously or to change the composition of the medium or the initial reaction.

The failure to observe any one of these factors may have been responsible for the contradictory statements found in the literature with regard to bacterial hydrolysis of starch. It is always more difficult to demonstrate the presence of amylase than its absence.

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STUDIES ON CARBON AND NITROGEN SOURCES FOR THE PRODUCTION OF AMYLOLYTIC ENZYMES BY SUBMERGED CULTURE OF *ASPERGILLUS NIGER*[1]

By P. Shu and A. C. Blackwood

Abstract

Aspergillus niger PRL 558 was used in the production of starch saccharifying enzymes. The culture was grown on 100 ml. of medium in 500 ml. Erlenmeyer flasks agitated and aerated on a rotary shaker at 35° C. Quantitative analyses for amylase, maltase, and limit dextrinase were determined on the culture filtrates. Variations in the specific type of carbohydrate source affected the yield of amylase markedly, and of maltase to a lesser extent. The production of limit dextrinase is the least dependent on the carbohydrate source. Maltose or compounds constituted of maltose units are essential for producing a maximum yield of amylase. The yields of the enzymes are related to the degree of the availability of the nitrogen source. Highest enzyme yields are obtained with hydrolyzed protein. However, the production of amylase and maltase is further stimulated by adding inorganic nitrogen compounds such as ammonium nitrate and sodium nitrate as a supplementary nitrogen source. The amounts of maltase and amylase obtained are controlled also by varying the fermentation time as well as the carbohydrate and protein content of the medium.

Introduction

Most of the earlier studies on the production of fungal amylases have been done in the preparation of "mold bran". This method was first developed by Takamine (20) and extensively studied by Christensen (4) and Underkofler and collaborators (23-27). Roberts *et al.* (17) and Hao and coworkers (10, 11) have also reported the successful use of the mold bran in grain saccharification.

Recently, the submerged culture method has received considerable attention. Workers at the Northern Regional Research Laboratory, Peoria, published a number of papers (6, 15, 21, 28) on the study of this method and the use of the enzymes produced. Adams *et al.* (1) and Erb *et al.* (7, 8) have also reported an investigation of the method.

With regard to the use of the fungal preparation, Corman and Langlykke (6) reported that the efficiency of alcoholic fermentation of grain was related to the maltase activity of the *A. niger* preparation, when the latter was used as conversion agent. However, Kneen and Spoerl (13) found that, with barley malt as the saccharifying agent, the alcohol yield of grain fermentation was related to the limit dextrinase activity of the malt. In the study of secondary fermentation of corn mashes converted by *A. niger*, Pan *et al.* (16) observed that the rate of the fermentation was related to maltase activity of the fungal preparation. Similarly, Cordon and coworkers (5) also noticed the significance of maltase activity of *A. niger* in producing sugar from potatoes

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for lactic acid fermentation. It seems likely that the proper balance of these enzyme activities (i.e. amylase, maltase, and limit-dextrinase) in fungal preparations is essential. A knowledge of the factors affecting the production of these enzymes is desirable.

So far, investigations on amylase production were all confined to the use of crude materials (e.g. thin stillage, distiller's dried-solubles, and corn mashes), with which the separate studies on basic variables are difficult. The purpose of this study is twofold: (1) to show the effect of variation of medium composition on the activities of maltase, amylase, and limit dextrinase of a submerged culture of *A. niger*, and (2) to describe the methods of producing different relative activities of amylase, maltase, and limit dextrinase with this same mold.

Methods and Materials

A preliminary screening program for high amylase-producing molds grown in submerged culture indicated that *Aspergillus niger* PRL 558 was one of the most active. This culture was obtained from the Division of Applied Biology, National Research Council, Ottawa, numbered A_1-23. In these studies this *A. niger* strain was used throughout. Fermentations were carried out in 500 ml. cotton-plugged Erlenmeyer flasks, each containing 100 ml. of medium. The basal medium contained 0.5% calcium carbonate, 0.1% potassium dihydrogen phosphate, and 0.05% magnesium sulphate septahydrate, to which various nitrogen and carbon sources were added. Temperature of fermentation was kept at 35° C. Five per cent of a 24-hr.-old vegetative inoculum was used in the experiments. Aeration and agitation were obtained by shaking the fermentation flasks on a rotary Gump shaker,* running at 206 r.p.m. Enzyme analyses were made after 72 hr. fermentation, or as otherwise stated.

Sugars were determined with modified Somogyi (19) reagent. Total and ammonia nitrogens were determined by micro method as described by Umbreit *et al.* (22). Glycine and glutamic acid estimations were made by Sørensen's Formol titration (12). Nitrate and urea were first converted to ammonia with Devarda's Alloy and urease respectively in a Conway microdiffusion cell, and the liberated ammonia was titrated against 0.1 *N* sulphuric acid (12). Glycerol was determined as described by Lambert and Neish (14). Maltase activity was determined by the method of Corman and Langlykke (6), while the limit dextrinase activity was determined by the method of Back *et al.* (2). The determinations were made under the conditions where the products of time and enzyme concentrations were constant. The unit activity of maltase was defined as mgm. of maltose monohydrate hydrolyzed in one hour, while the unit activity of limit dextrinase was expressed as mgm. of limit dextrin (according to Kneen) hydrolyzed per hour, both at 30° C. Alpha-amylase was determined by the method as described by Blackwood (3). Its unit activity was defined as mgm. of Lintner starch hydrolyzed to an arbitrary

* *A product of B. F. Gump and Company, Chicago, Ill.*

starch–iodine end point in one hour, at 30° C. One thousand units of the amylase activity thus determined was found to be equivalent to 3.7 units of α-amylase determined according to the method of Sandstedt, Kneen, and Blish (18). No evidence of the production of β-amylase by this strain of *A. niger* was observed; therefore, no β-amylase treatment of starch was made.

Chemicals used in the experiments were all the best commercial products available.

Results and Discussion

Non-nitrogenous Carbon Source

LeMense and his associates (15) studied the effect of a number of carbon sources supplemented to corn steep liquor, dry tankage, soy bean meal, and thin stillage on amylase production. They found that corn meal was highly satisfactory. Their results indicate that the type of the supplementary carbon sources influences the yield of amylase. Recently, Goodman (9) obtained amylase from fungi grown in media containing different proportions of sucrose and starch, and observed that the yield depended upon the starch content of the medium. From this evidence, he concluded that the amylase production by fungi is adaptive.

In a preliminary study, it was found that proteins did not support growth of the organism used in the present investigation. Therefore, the use of a supplementary carbon source is necessary. A number of types of organic compounds, namely, polyalcohols, acids, and carbohydrates, have been tried. The fermentations were conducted under the described conditions. The media contained 1.5% hydrolyzed isoelectric casein as the sole source of nitrogen. The amounts of non-nitrogenous carbon compounds added to the media were equivalent to 2% glucose (on carbon basis). The experimental results are given in Table I. Of all the substances tried, only galactose, lactose, mannitol, α-methylglucoside, and sodium gluconate did not support satisfactory growth. The enzyme activities of culture filtrates varied widely depending upon the carbon source present. Maltose or compounds composed of maltose units gave highest yields of the enzymes. Sucrose or fructose gave the best growth but not the maximum enzyme yields. Thus, abundant growth seemed to be a necessary but not sufficient condition for maximum production of these enzymes. Comparing these three enzymes, the production of limit dextrinase is the least dependent on the substrate while amylase production was very sensitive to the type of the substrate present. Higher maltase activities were obtained with glucose or substances containing glucose units. There is a possibility that in the presence of a readily oxidizable substrate, such as glucose and xylose, the local pH values of the mycelia are sufficiently low to hamper amylase production; but this is ruled out by the fact that fructose, glycerol, and lactate did not give high yields of the enzyme. Since these latter substrates are either very slowly utilized by the organism

or are not potential acid formers, little acid accumulation is expected in the fermentations with these substances. These results seemed to favor the theory that amylase production is adaptive.

TABLE I

EFFECT OF NON-NITROGENOUS CARBON SOURCES ON THE PRODUCTION OF THE ENZYMES

Compounds	Mycelial growth, gm./100 ml.	Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.
Starch	2.04	1145	23.0	26.5
Dextrin	—	1060	18.8	—
Maltose	1.96	1210	24.7	25.7
Glucose	1.80	339	14.7	18.8
Fructose	2.60	317	7.3	18.5
Sucrose	2.40	366	10.4	21.0
Xylose	1.74	66	2.4	18.0
Galactose	Trace	80	2.0	—
Lactose	0.45	170	10.4	—
Glycerol	1.50	88	4.0	16.5
Lactic acid*	1.30	83	4.1	18.5
Mannitol	Trace	16	0.0	—
α-Methylglucoside	Trace	70	0.0	—
Gluconic acid*	Trace	20	0.0	—

** The initial pH of the medium was adjusted to 5.5 with dilute sodium hydroxide solution.*

NOTE: *Fermentation medium: basal plus 1.5% casein hydrolyzate plus the amounts of carbon compounds equivalent to 2% glucose (on carbon basis).*

The specificity of the carbohydrate structure for amylase production was further studied in media containing 3% carbohydrates (see Table II). The synthesis of amylase was significantly stimulated when one-third of the glucose in a 3% glucose medium was replaced by maltose; and the adaptation seemed to be specific to the maltose structure since the substitution of maltose

TABLE II

SPECIFICITY OF THE CARBOHYDRATE STRUCTURE FOR AMYLASE SYNTHESIS

Medium contents of carbohydrates or their derivatives	Mycelial growth, gm./100 ml.	Amylase yield, units/ml.
3% Maltose	1.85	1300
1% Maltose and 2% glucose	1.98	980
3% Cellobiose	2.53	487
1% Cellobiose and 2% glucose	2.11	620
1% Lactose and 2% glucose	1.86	610
1% α-Methylglucoside and 2% glucose	1.73	162
3% Glucose	1.67	353

NOTE: *Fermentation media: basal plus 1.5% casein hydrolyzate plus 3% carbohydrates or their derivatives.*

by structurally related compounds (i.e. cellobiose, α-methylglucoside, lactose) in the medium did not give a comparable amylase yield. If maltose were hydrolyzed in the initial phases of the fermentation, then it is difficult to explain by the adaptation theory the high production of amylase. However, it has been found that under present experimental conditions all the maltose in a 3.5% solution was assimilated within 24 hr.; and no accumulation of free glucose was found during the course of the fermentation (see Table III). Thus it is shown that maltose effectively serves as an adaptive substrate for amylase synthesis by the organism.

TABLE III

CHANGES IN NON-NITROGENOUS CARBON SOURCES DURING THE FERMENTATION

Carbon source	Residual carbon source							
	0 Hr.		12 Hr.		24 Hr.		36 Hr.	
	Gm./ 100 ml.	% Hydro-lyzed*	Gm./ 100 ml.	% Hydro-lyzed	Gm./ 100 ml.	% Hydro-lyzed	Gm./ 100 ml.	% Hydro-lyzed
Glucose	3.9	—	2.0	—	0.0	—	—	—
Fructose	3.5	—	2.5	—	—	—	1.6	—
Sucrose	3.8	0.0	2.0	97.0	0.85	—	0.04	—
Maltose	3.4	0.0	2.5	0.0	0.14	0.0	—	—
Glycerol	2.9	—	—	—	2.7	—	2.5	—

** The % hydrolysis of the residual carbohydrate was calculated after determining reducing power before and after acid hydrolysis.*

NOTE: *Fermentation media: basal plus 1.5% casein hydrolyzate plus amounts of carbon compounds as indicated.*

Nitrogen Source

It is generally believed that proteinaceous substances present in crude materials, such as corn steep liquor or thin stillage, serve as nitrogen source for the growth of the organism. As yet, it has not been clearly shown what type and what form of nitrogen source would give maximum efficiency in the production of the enzymes.

In the present study, four types of nitrogen source (nitrates, amides, ammonium and amino compounds) were investigated. Three per cent Lintner soluble starch was used in the media as the non-nitrogenous carbon source. Fermentations were carried out under regular conditions, except that pH values of the culture solutions were examined at 12-hr. intervals and kept above 4.8 by adding 0.25 gm. amounts of calcium carbonate, when necessary. The experimental results summarized in Table IV show that amides (such as urea and acetamide) do not support good growth and are slowly utilized. None of the single nitrogen compounds give yields of enzymes comparable to casein hydrolyzate (on equal nitrogen basis). However, inorganic nitrogen

TABLE IV

THE PRODUCTION OF THE ENZYMES FROM VARIOUS NITROGEN SOURCES

Nitrogen source	Quantity added, gm./100 ml.	With no casein hydrolyzate					With 1.5% casein hydrolyzate			
		Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.	Mycelial weight, gm./ 100 ml.	Nitrogen uptake, mgm./ 100 ml.*	Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.	Mycelial weight, gm./ 100 ml.
Ammonium chloride	0.78	40	14.0	15.8	1.32	38	2880	41.6	30.0	1.56
Sodium nitrate	1.26	144	18.6	13.5	1.75	32	1910	42.0	29.5	1.69
Ammonium nitrate	0.60	12	5.6	9.0	1.38	49	2740	40.8	30.0	1.43
Urea	0.43	225	4.4	15.8	0.98	16	1785	10.0	15.4	0.93
Acetamide	0.84	20	2.8	—	(Poor)	—	—	—	—	—
Asparagin	0.97	12	2.5	—	—	—	—	—	—	—
Glycine	1.27	12	4.4	3.5	1.63	48	—	—	—	—
Glutamic acid	2.10	228	10.0	9.6	2.30	47	—	—	—	—
Casein hydrolyzate	1.50	1642	27.6	30.5	1.74	77	—	—	—	—

* *Nitrogen uptake by the organism in first 24 hr.*

NOTE: *Fermentation media: basal plus 3% soluble starch plus nitrogen source as indicated.*

compounds (such as ammonium chloride, ammonium nitrate, and sodium nitrate), when added to media containing casein, stimulated the production of amylase and maltase (see Table IV). This effect is less prominent when the protein concentration is increased (Table V). The type of protein employed definitely affects the production of the enzymes. This is probably

TABLE V

THE USE OF AMMONIUM NITRATE AS SUPPLEMENTARY NITROGEN SOURCE

Protein		Ammonium nitrate added, gm./100 ml.	Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.
Type	Quantity, gm./100 ml.				
Whole casein	1.5	—	380	8.2	14.0
Whole casein	1.5	0.6	986	17.2	22.5
Casein hydrolyzate	1.5	—	1672	27.6	30.0
" "	1.5	0.6	2735	37.0	35.0
" "	6.0	—	2160	39.2	26.2
" "	6.0	0.6	2760	45.6	26.0

NOTE: *Fermentation media: basal plus 3% soluble starch plus amounts of casein and ammonium nitrate as indicated.*

not a result of differences in the amino acid contents of the proteins since their hydrolyzates all gave high enzyme yields (see Table VI). This experimental evidence and the data on utilization rates (Table IV) strongly suggest that the availability of the nitrogen source to the organism is one of the most important factors to be considered in the production of the amylolytic enzymes.

TABLE VI

A COMPARISON OF WHOLE PROTEINS AND THEIR HYDROLYZATES IN THE PRODUCTION OF THE ENZYMES

Name	Whole protein			Hydrolyzate*		
	Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.	Amylase, units/ml.	Maltase, units/ml.	Limit dextrinase, units/ml.
Casein	562	9.0	18.9	1492	26.0	31.0
Gelatin	250	4.4	14.2	1920	26.0	31.0
Gluten	34	3.3	None	2025	29.5	28.5
Edestin	27	6.6	None	1385	23.0	31.5

* *The proteins were hydrolyzed with 0.1% Rhozyme (obtained from Rohm and Haas Co., Philadelphia).*

NOTE: *Fermentation media: basal plus 3% soluble starch plus 2% proteinaceous substance.*

The Control of Enzyme Yields by Variation of the Carbohydrate and Protein Concentrations

It is often desirable to have preparations containing varied relative concentrations of the enzymes. With a medium containing low concentrations of distiller's dried-solubles, Tsuchiya and associates (21) were able to obtain a culture solution containing high maltase and low amylase by increasing the corn meal content of the medium. In the present study, similar results were obtained by increasing the starch content of the medium containing low concentrations of casein hydrolyzate (Fig. 1). This indicates that no other factors present either in corn meal or distiller's dried-solubles are required for the production of a high maltase and low amylase preparation.

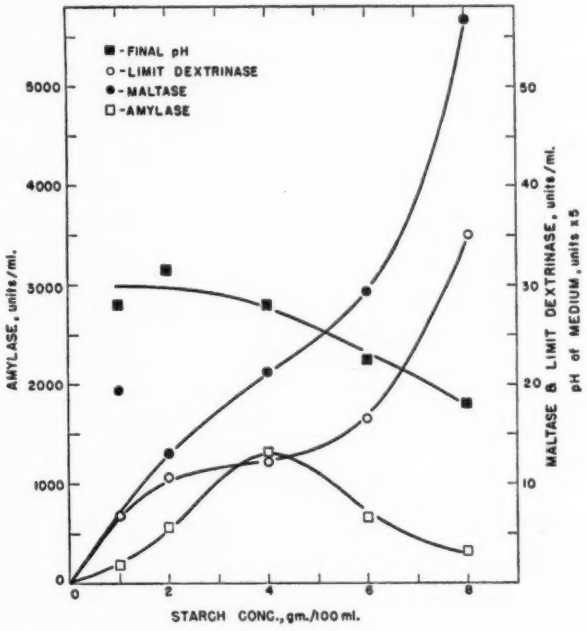

FIG. 1. The effect of variations in starch concentrations in low protein media on the production of the enzymes. All fermentation media contained 1% casein hydrolyzate. Determinations of pH and enzyme activities were made after 96 hr. of fermentation.

The significance of the fermentation time as well as the relative carbohydrate and protein concentrations on the yields of amylase, maltase, and limit dextrinase was also demonstrated. It is found that an increased amount of either carbohydrate or protein increases the time required to attain the maximum amylase yield, and also increases the maximum yields of all three amylolytic enzymes (Table VII). In protein carbohydrate unbalanced media,

TABLE VII

VARIATIONS OF STARCH AND CASEIN HYDROLYZATE CONCENTRATIONS AND THE EFFECT ON MAXIMUM YIELDS OF THE ENZYMES

	Fermentation time to reach maximum yield, hr.			Maximum enzyme yield, units/ml.		
	Amylase	Maltase	Limit dextrinase	Amylase	Maltase	Limit dextrinase
A. 2% Casein hydrolysate						
Starch, gm./100 ml.:						
1.0	48	144	192	785	29.4	20.7
2.0	72	144	192	1830	35.4	24.7
4.0	96	144	192	3480	40.0	30.0
6.0	96	144	168	3220	48.8	31.0
B. 2% Soluble starch						
Casein hydrolyzate, gm./100 ml.:						
0.5	72	192	192	892	14.7	5.9
1.0	72	192	192	1190	37.6	14.4
2.0	96	144	144	1830	42.4	24.7
4.0	96	144	192	3250	37.6	28.2
6.0	144	144	192	2280	39.0	40.6

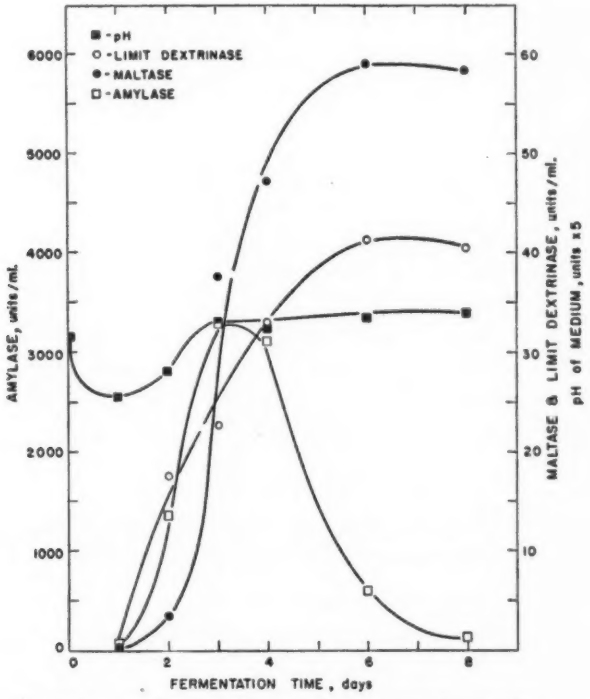

FIG. 2. Destruction of amylase by prolonged fermentation. Fermentation medium contained 2% starch and 6% casein hydrolyzate.

the peak of maximum yield of amylase occurs earlier than those of maltase and limit dextrinase in the course of fermentation (see Table VII). In general, a rapid amylase destruction occurs after the maximum yield is reached; such destruction is not necessarily a pH effect. By taking advantage of this phenomenon, a culture solution containing low amylase but high maltase and high limit dextrinase may be prepared. An example of this technique is shown in Fig. 2.

A low maltase preparation was obtained by a short fermentation in a medium containing both high carbohydrate and protein. The significance of the fermentation time is demonstrated by comparing Figs. 3A and 3B.

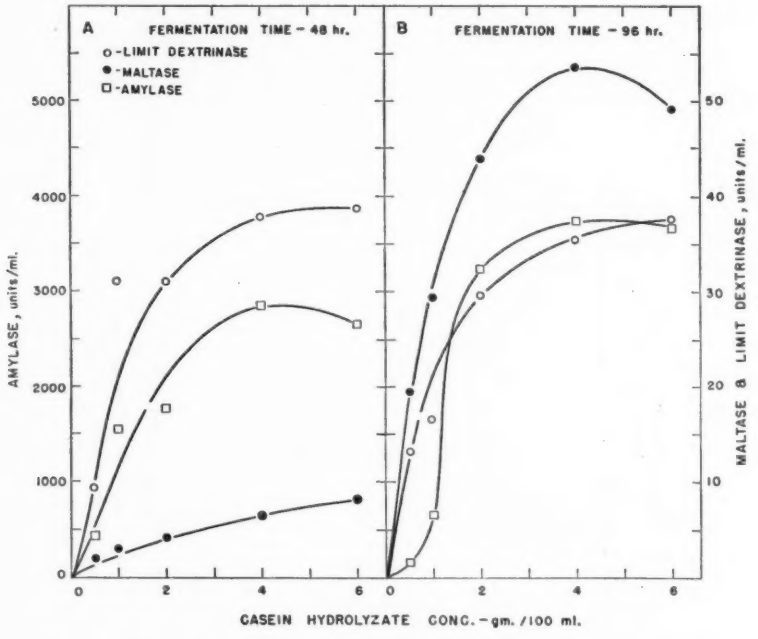

FIG. 3. Preparation of low maltase culture solution with a short fermentation. Fermentation medium contained 3% soluble starch.

Fig. 4 represents a typical fermentation which produced high yields of maltase, amylase, and limit dextrinase. The production of preparations containing low limit dextrinase with a high maltase and amylase activity was not successful because of the stability and less specific conditions required for the synthesis of the limit dextrinase.

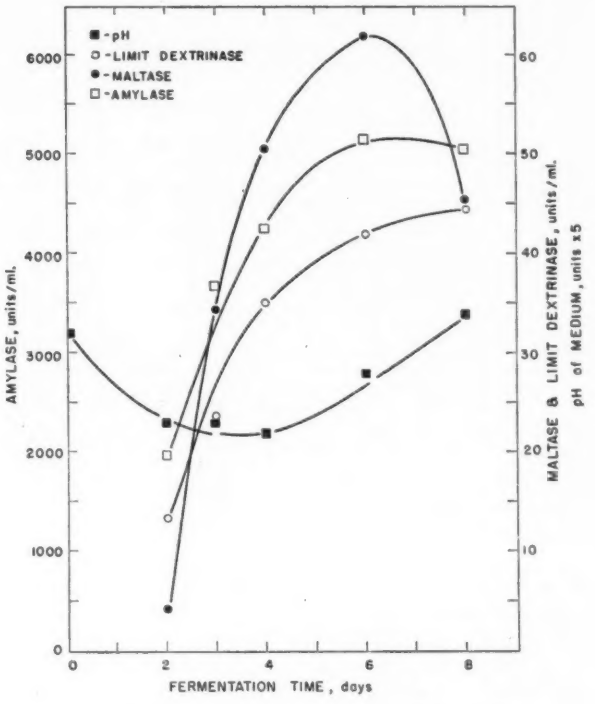

FIG. 4. Production of high yields of amylolytic enzymes.
Fermentation medium contained 8% starch and 4% casein hydrolyzate.

Acknowledgment

The authors wish to express their appreciation to Dr. Wendy M. Dion for her valuable advice and for supplying the cultures used in this investigation. The technical assistance of J. M. Pringle is gratefully acknowledged.

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A PRELIMINARY STUDY OF CORN SEEDLING BLIGHT IN SOUTHERN ONTARIO[1]

By W. E. McKeen[2]

Abstract

In southern Ontario, seedling blight is the major factor responsible for poor stands of corn and its gradual decline in yield for the past number of years. This disease expresses itself as an uneven stunting and chlorosis throughout the field. It may be caused by *Pythium arrhenomanes* Drechs., *Pythium debaryanum* Hesse., *Fusarium moniliforme* Sheld., *Fusarium graminearum* Schw., or *Rhizoctonia solani* Kuehn. *P. arrhenomanes*, which is the most economically important pathogen encountered, produces millions of zoospores in moist soil. Under natural field conditions, it seldom kills the plant, since corn possesses unusual capacity to send out new adventitious roots when the causal organism destroys the older roots. In the course of these studies, *F. graminearum* was found only once and apparently is not important as a soil-borne organism in this corn–wheat-growing area. *F. moniliforme* was present in most lesions, but it is not an active root parasite and, in the majority of cases, is a secondary invader. Studies conducted in refrigerated rooms have shown that temperature has a greater influence on the hosts (wheat and corn) than on the organism, *P. arrhenomanes*, because disease incidence was greatest at low temperatures for corn and at high temperatures for wheat. Although nematodes are very numerous in necrotic roots, they appear to be of little importance.

Introduction

In Ontario, corn is grown throughout the southern part and this region is actually an extension of the large corn belt that includes Indiana, Illinois, Iowa, Ohio, Nebraska, Wisconsin, Minnesota, and the Dakotas. The northern boundaries are gradually extending into the cooler regions owing to the development of earlier maturing hybrids, and good crops are being obtained even as far north as Carleton County.

Of late years, approximately 75% of the total acreage of corn in Canada has been concentrated in Ontario and, in 1949, well over 600,000 acres were produced. Of this, 272,000 acres consisted of shelled corn, which was grown mostly in Essex, Kent, Lambton, Middlesex, and Elgin counties, and was valued at approximately 20 million dollars. The ensilage corn, which constitutes the remaining acreage, is the basic food for thousands of dairy and beef herds, the food value obtained from one acre of corn being much greater than that from any other forage crop.

Although corn in Ontario may suffer from many diseases, at the present time root rot is the most widespread and economically significant pathological condition with which the grower has to cope. Since practically all seed corn is treated with Arasan, or one of the other newer organic fungicides, seedling emergence is ensured if healthy seed is planted and seed decay is no longer a factor, because these fungicides destroy the true ear rot organisms (*Diplodia*

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zeae, *Fusarium graminearum*, and *Fusarium moniliforme*) which may adhere to the seed, and species of *Aspergillus*, *Penicillium*, and *Rhizopus* which may be extremely destructive on injured kernels.

Root rot becomes apparent during seedling development and is the major factor responsible for poor stands of corn and a gradual decline in yield during recent years. This disease only occasionally destroys a field completely, but stunting, chlorosis (Figs. 1 and 2), and sometimes reddening of affected tissues are common symptoms, because the corn plant has a remarkable capacity to send out new adventitious roots as its primary, seminal, and adventitious roots are destroyed by the pathogenic organism. Owing to stunting of affected plants, diseased fields present an uneven appearance. Stunting is usually most noticeable in the low regions on the clay soils, but the reverse is frequently true on certain sandy soils. The influence of previous crops has also been observed. This is clearly shown in Fig. 3, where one-half of the corn field, which was in tobacco three years previously, is tall and healthy, and the remaining half, which was in corn at that time, is short and stunted. During the two intervening years, when oats was followed by clover, both portions of this field received similar treatment. Since affected plants usually survive a root rot attack, disease damage is most difficult to determine, but in many instances yield reductions of more than 50% have been indicated.

The present study was undertaken to determine the identity and relative importance of the causal organisms responsible for root degeneration in corn and to study the factors influencing the incidence and severity of such diseases as might be found in Ontario.

Literature Review

The earlier work on corn root rot is summarized by Manns and Philips (11) in 1924: "The literature as here reviewed shows that investigators have determined upon four different organisms as the principal ones associated with corn-rot diseases. These organisms are *Fusarium moniliforme* Sheldon; *Gibberella saubinetii* (Mont.) Sacc.; *Diplodia zeae* (Schw.) Lev., and *Cephalosporium sacchari* Butler. It is known that several of these organisms inhibit germination and appear to be active factors in the production of stalk and ear rots. Their importance as factors in causing rootrot, however, is still in dispute. It is even questioned whether soil reaction is not the real factor predisposing corn to attacks of these so-called seedling and rootrot diseases." In 1923, Hoffer and Carr (4), working in Indiana, were convinced that the root rot condition in corn was associated with soil deficiencies and toxicity of aluminum and iron concentrations.

At the same time Koehler and his associates (8) maintained that the organisms that caused stalk and ear rots were also able to infect and rot the roots when introduced into the ground by means of diseased seed. They also stressed the fact that, since corn and wheat are both subject to the ravages of *Gibberella saubinetii*, these crops should not follow each other in a rotation.

In Illinois, Tehon (13), in 1924, found that root and stalk rots of corn were caused chiefly by the fungi *F. moniliforme*, *Diplodia zeae*, and *G. saubinetii*, and that the degree of injury done by these organisms was dependent upon the relative susceptibility of the corn plant in the various stages of its development.

In 1932, Leonian (10) stated that *F. moniliforme* was not an active root parasite and that, at least in the majority of cases, it was a secondary invader. In Valleau's (15) earlier studies, he considered *F. moniliforme* to be the most important agent in root and stalk rots of corn, but later (16) found that a *Pythium* fungus was of major importance. He questioned the importance of *G. saubinetii*, because in more than 100 isolations made from rotting corn roots of plants growing in the field, in pot cultures of diseased soil, or in sand cultures inoculated with rotten corn roots, this organism was not obtained.

In 1930, Koehler and Holbert (9) differentiated between the rots due to seed infection, which are confined to an area rather close to the seed, and the more widespread root rot, which is not caused by seed infection but rather by organisms occurring in the soil.

More recently, Elliott (2) isolated *Pythium arrhenomanes* from specimens of inbred and open-pollinated field corn from Iowa and Nebraska and has indicated that this root rot continues to have an important effect on the yield of corn. Wen-Chun Ho (5) has indicated also that this organism is important, but that there appears to be a succession of invasions by different species of fungi as the season advances. Richardson (12) reported that root rot of corn was caused primarily by parasitic soil microorganisms and that species of *Pythium*, *Helminthosporium*, and *Fusarium* were the most important.

Survey of Pathogens Associated with Seedling Blight

To determine the organisms responsible for root decay, isolations were made from roots of diseased seedlings grown in soil obtained from widely separated commercial fields in which seedling blight had been severe. The healthy seed which was planted in these soils was treated with Arasan in order to eliminate seed-borne organisms and to provide conditions similar to those in commercial fields. The resulting seedlings were grown at different constant temperatures, and roots that showed incipient lesions were washed thoroughly in running water before they were placed in Petri plates containing water or agar media. Potato sucrose or dextrose agar was used throughout in order that *Fusarium graminearum* could be detected readily by its red stroma. The following fungi were obtained: *Pythium arrhenomanes* Drechs., *Pythium debaryanum* Hesse., *Fusarium moniliforme* Scheld., *Fusarium graminearum* Schw., *Rhizoctonia solani* Kuehn, *Penicillium* spp., *Actinomyces* spp., *Mucor* spp., *Trichoderma* spp., and certain others that were not identified.

Species of *Pythium* and *Fusarium* were obtained most frequently and in a great many instances they grew out of the same lesion. Only a single culture of *F. graminearum* was obtained, even though a wheat–corn rotation, which

is considered favorable to the development and survival of this organism, is practised by many farmers. Bacteria were always present, but were of no primary importance. The results in Table I are quite representative and

TABLE I

RELATIVE FREQUENCY OF FUNGI ISOLATED FROM ROOTS OF DISEASED CORN SEEDLINGS GROWN IN ROOT ROT SOIL

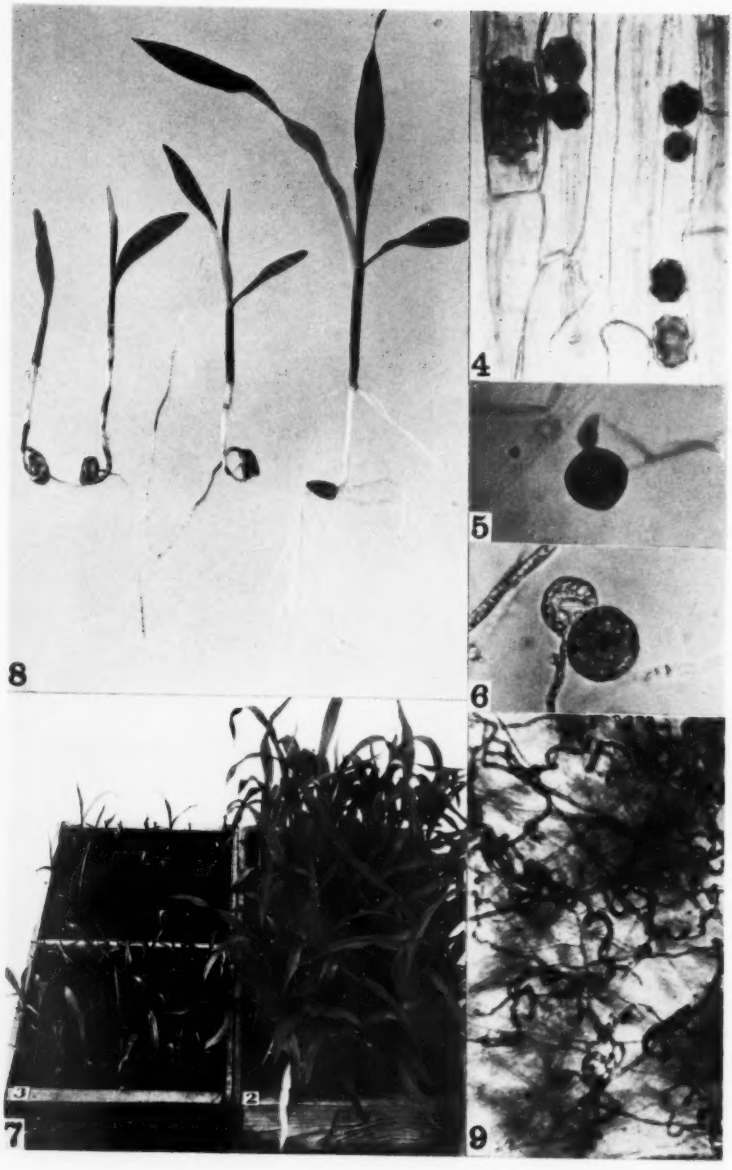
Fungi	Number of isolates obtained			
	King's Brookston clay	Winter's Brookston clay	Brian's sandy loam	Total
Pythium spp.	116	134	73	323
Fusarium spp.	109	131	78	318
Fusarium graminearum	0	1	0	1
Rhizoctonia solani	9	4	13	26
Mucor spp.	6	8	2	16
Penicillium spp.	7	1	4	12
Actinomyces spp.	5	4	3	12
Trichoderma spp.	2	4	3	9
Unidentified spp.	13	8	8	29

similar to those obtained from decayed roots taken from other locations. Pathogenicity tests were conducted by planting hybrid seed corn in sterile soil inoculated with cornmeal–sand cultures of all the above-mentioned organisms. Of the numerous organisms tested, *Pythium arrhenomanes*, *Pythium debaryanum*, *Fusarium moniliforme*, *Fusarium graminearum*, and *Rhizoctonia solani* proved to be pathogenic.

Numerous microscopic observations of infected roots stained in acid fuchsin lactophenol showed that both *Pythium* and *Fusarium* spp. were present in most of them, but only phycomycetous mycelium was detected in the smallest lesions and at the advancing edges of the older ones. In the older part of the lesion, *Pythium* sporangia, *Fusarium* mycelium, and nematodes were quite numerous and, in central regions, oogonia and sometimes *Asterocystis* sp. could be observed (Fig. 4). *R. solani* was frequently observed adjacent to the roots or rootlets, but occasionally it penetrated the epidermal cells and filled them with its closely segmented mycelium. To judge by the relative frequency of isolation, microscopic observations, and disease symptoms observed both in the field and under controlled conditions, *P. arrhenomanes* appeared to be the most virulent and economically important of the pathogens encountered, and for this reason it was studied in some detail.

FIG. 1. A field of corn seedlings suffering from severe root rot. Note the stunting and unevenness of the plants. FIG. 2. Flats of corn grown in 'root rot' soil. The soil in the one on the right, however, was steam-sterilized previous to planting. FIG. 3. The influence of previous crops is clearly shown in this field. The part on the left grew tobacco and the one on the right produced corn three years previously; oats followed by clover was grown in the whole field during the intervening period.

PLATE I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The virulence of all the pathogens which, in the meantime, were maintained in sand cultures, was compared in refrigerated rooms kept at 24°, 20°, 16°, and 12° C., under artificial lighting. In each flat, which contained steam-sterilized compost, 100 cc. of cornmeal–sand inoculum was added and, in each half of the flat, 40 Arasan-treated seeds of the inbred lines A and W26 were planted. Three naturally infested flats of soil were also planted in each room in order that symptoms produced by the pathogens might be compared with those occurring under natural conditions.

Pythium Root Rot

In all, four *Pythium* spp. have been observed attacking corn seedlings in Ontario, but three of them are of minor importance and one, which possesses spiny oogonium, has been observed only twice in the roots and has not been identified. *P. debaryanum* (Figs. 5 and 6) has proved to be widespread but not very virulent and only causes a slight amount of stunting even under ideal conditions. *P. aphanidermatum*, which is responsible for corn stalk rot during excessively hot wet periods, has at no time been isolated from seedling roots in Ontario under natural conditions, though this fungus proved to be capable of inducing root rot at temperatures above 30° C.

Symptoms

The most apparent symptoms of root rot caused by *P. arrhenomanes* was stunting and dwarfing (Fig. 7), which symptoms on plants growing in moist, heavily inoculated soil were apparent even at 24° C. A reddening of the basal sheaths and chlorosis of the leaves were very common, but, on plants about one foot high, a reddening of the upper leaves was sometimes observed. At 20° C., the plants did not grow more than 3 in. in height, but were, nevertheless, able to survive for about one month. This disease became more severe in the roots maintained at temperatures below 20° C., and at 12° C. there was no emergence.

Points of infection were scattered along the roots (Fig. 8), but tended to be concentrated at the tips. Incipient lesions appeared as brown specks, but necrosis soon spread rapidly along the root and severed it. The lesions were clear, flaccid, and water-soaked, with the exception of the advancing edges which were a dark, brownish-yellow color. This fungus frequently advanced upwards into the aboveground portions of the adventitious roots, but the first internodes were attacked only under extreme conditions.

FIG. 4. *Asterocystis* sp., in cortical and epidermal tissue of a diseased corn root. FIG. 5. An oogonium and a monoclinous antheridium of *P. debaryanum*. FIG. 6. A diclinous antheridium attached to an oogonium of *P. debaryanum*. FIG. 7. Flats of corn seedlings grown in sterilized soil but No. 3 was inoculated with *P. arrhenomanes*. Notice the stunting and dwarfing. FIG. 8. Seedlings showing symptoms of root rot caused by *P. arrhenomanes*. From right to left: healthy; watersoaked lesion on root; only short roots remaining and several lesions on the mesocotyl; no roots remaining and a section of mesocotyl completely necrotic, leaves have not unfolded. FIG. 9. Lobulate mycelial complexes with sporelike bodies attached.

Life History of Pythium

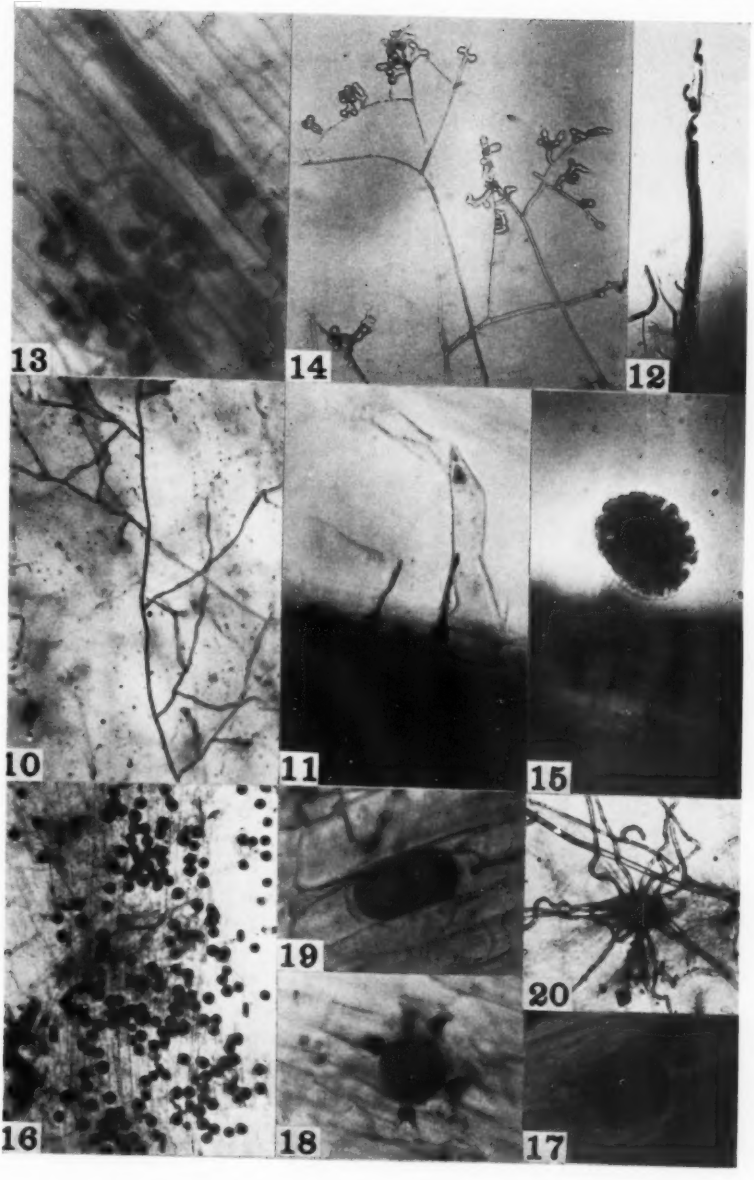
The nonseptate mycelium, which may be intra- or intercellular, is about 2.0 to 5.5 μ in diameter, with the exception of the constricted portion which penetrates the cell walls and the enlargements on either side. On agar media, structures which resemble single spored, elongated basidia (Fig. 9) begin to form after a week's development. These structures, as well as the dichotomous branching of the lateral branches and the weak spiral of the secondary branches (Fig. 10), aid in the identification of this organism. Vanterpool *et al.* (17) believe the former structures are appressoria.

Asexual reproduction can be induced without difficulty by immersing in water roots that are infected with *P. arrhenomanes*. In a short time numerous evacuation tubes, up to 75 μ long and 3 to 4 μ in diameter, may be seen emerging from epidermal cells (Fig. 11) or root hairs. These join up with lobulate, inflated, filamentous sporangia (Figs. 12 and 13), which may fill many of the epidermal or cortical cells in the clear zone behind the advancing mycelium. The few sporangia (Fig. 14) which are formed on the mycelium which grows out into the water, are morphologically distinct from those formed within the cells. They have several short, inflated branches and resemble the asexual organs of *P. aphanidermatum*.

The tip of the efferent tube gradually enlarges as the protoplasm from the sporangium moves into the vesicle (Fig. 15). Individual zoospores are delimited frequently before all of the protoplasm has flowed into the vesicles, which range from 5 to 40 μ in diameter and contain from 2 to 38 zoospores, with an average of 17.3. In about five minutes, movement of the individual zoospores begins, and soon a vigorous lashing of flagella can be readily detected. Soon afterwards, the zoospores jostle violently within the vesicle, and three or four minutes later the vesicular membrane breaks. The zoospores, which are approximately 15 μ long and 8.5 μ wide and have two lateral flagella, escape in rapid succession and swim swiftly in all directions. They do not become motile in solutions with a pH lower than 5.

After one or two days, asexual reproduction ceases, except for a few sporangia which form on the mycelium extending out into the water. The sexual organs develop further back in the lesion and are frequently produced on artificial media, though certain isolates have been observed to remain sterile on the same media. Usually, however, hundreds of oogonia, averaging 30 μ in diameter, may be observed in diseased root tissues (Figs. 16, 17, 18)

FIG. 10. *P. arrhenomanes* mycelium showing dichotomy of the lateral branches and a weak spiral of the secondary branches. FIG. 11. Evacuation tubes that connect with the sporangia within the host cells. FIG. 12. A sporangium filling a corn root hair. FIG. 13. Several lobulate sporangia in the epidermal and cortical cells. FIG. 14. Sporangia formed externally in water. They assume a different shape to the ones formed within the host. FIG. 15. A vesicle just as the zoospores are beginning to be delimited. FIG. 16. Numerous oogonia in a corn root. FIG. 17. An oogonium containing an oospore. FIG. 18. Four antheridia attached to an oogonium. FIG. 19. An oogonium flattened owing to cell wall pressure. FIG. 20. A potential oogonium with several germ tubes.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and frequently, owing to cell wall pressure, they become flattened and average 35 μ by 17 μ in size (Fig. 19). It has proved difficult to detect the antheridia, which are, nevertheless, numerous, crook-necked, 12 to 25 μ long by 6 to 9 μ wide and make narrow contact with the oogonium, because they degenerate so rapidly. The oospores are plerotic, approximately 28 μ in diameter, with a wall about 1.5 μ thick, and they contain a single reserve globule. Sometimes a potential oogonium produces several germ tubes (Fig. 20).

Epidemiology

A combination of low soil temperature and abundant moisture are necessary to induce an epidemic of root rot. The necessity of abundant moisture is apparent, since only under such conditions could zoospore production, liberation, and dispersal occur. An examination of the growth–temperature curve, as shown in Fig. 21, and disease incidence, clearly indicates that pathogenicity

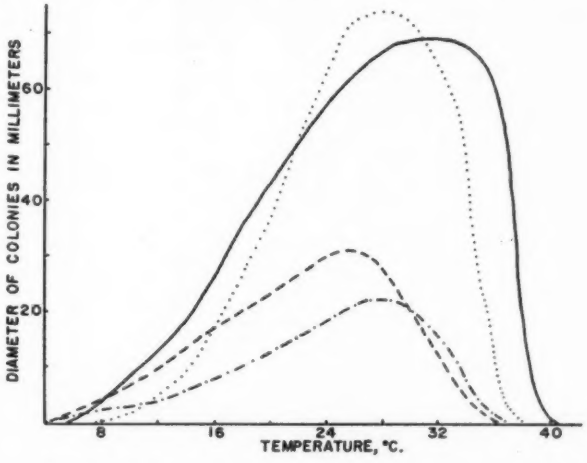


FIG. 21. Influence of temperature on the rate of growth of the four pathogens on potato dextrose agar: *P. arrhenomanes* ——————; *R. solani* · · · · · · · · ·; *F. graminearum* – – – – – – – – –; *F. moniliforme* —·—·—·—·—·—.

cannot be correlated with the rate of growth of the pathogen. The disease becomes less severe as the temperature is raised up to 30° C., and, at the same time, the greatest vegetative development of *P. arrhenomanes* occurs as the temperature approaches 32° C. The converse proved to be true with wheat. At 24° C., the wheat seedling check plants, 15 days after planting, were at least twice as high as those in the inoculated soil and the weights of the tops were 63 and 39 gm., respectively, whereas at 16° C. seedlings grew almost equally well in both infested and noninfested soil. This indicates that

temperature has a greater effect on the host than on the organism. The effect of temperature on the disease incidence in timothy, oats, and barley, all of which are susceptible, has not been tested.

Fusarium moniliforme Blight

In Table I, all of the *Fusarium* spp., with the exception of *F. graminearum*, were grouped together because it was impossible to be certain of the species unless considerable time and care were taken for its identification. Confirmation of the identity of the isolate of *F. moniliforme* used in these experiments was obtained from Dr. W. L. Gordon, Winnipeg. Symptoms of the disease caused by this fungus consist of yellowing of the leaf tips, followed by wilting of the lower leaves and then a sudden collapse of the plant. Frequently white mycelium develops on the water-soaked part of the stem at the surface of the soil. In contrast with *Pythium*, the locus of infection with *F. moniliforme* is usually in the crown and subcrown internode (Fig. 22). This explains the collapse of the seedling soon after infection.

From Table II, it will be observed that *F. moniliforme* caused only a small amount of pre-emergent damping-off even at 12° C., the lowest temperature used. At 24° C., disease was completely absent but, as the temperature was

TABLE II

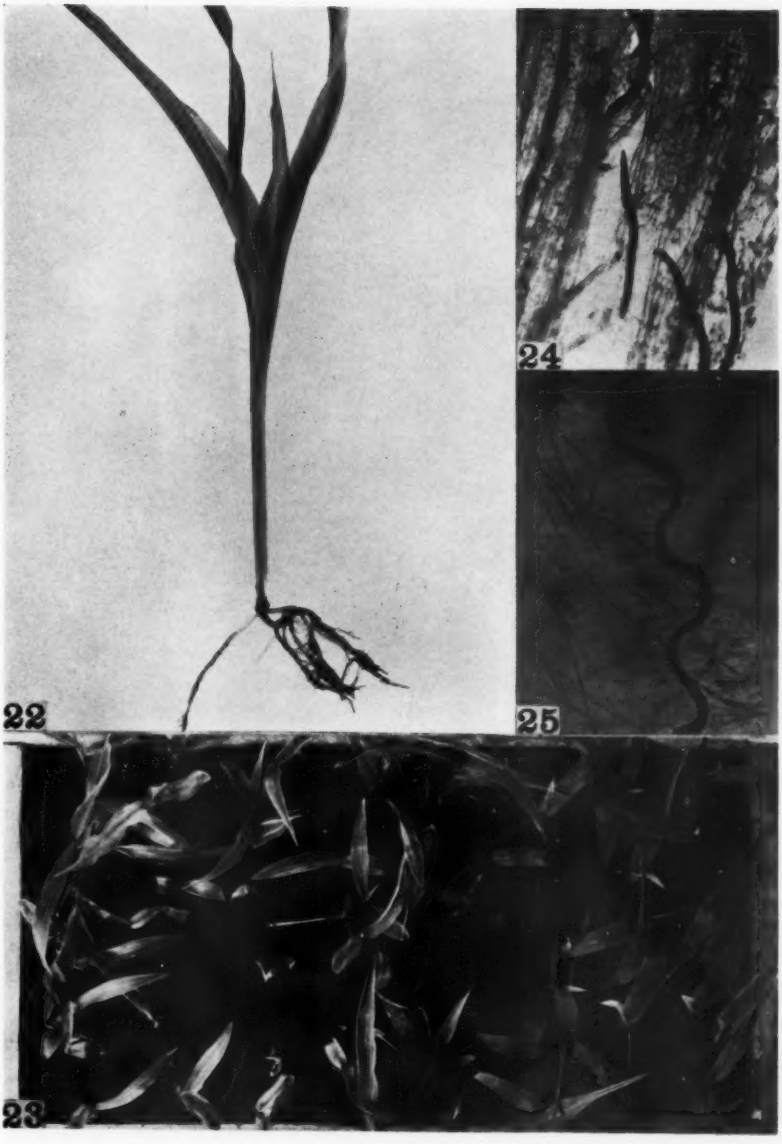
Effect of temperature on the incidence of seedling blight in sterile soil inoculated with *F. moniliforme**

Temperature	First appearance of blight	Percentage		
		Emergence	Postemergence blight	Final stand
24	—	95.0	0.0	95.0
20	25**	95.0	53.9	51.3
16	25	90.0	62.5	33.8
12	40	81.3	13.8***	70.0

* *Forty-four days after planting.*
** *Days after planting.*
*** *Postemergence blight just beginning.*

lowered, the incidence and severity of disease increased, though a long period was required before symptoms became evident. Since under field conditions the temperature does not remain at 12° or 16° C. for long periods, it is doubtful whether this organism could become important unless a fluctuating temperature would affect the host–parasite relationship very differently.

Fig. 22. A corn seedling infected with *F. moniliforme;* the roots are healthy but the crown is necrotic. Fig. 23. A flat of corn seedlings inoculated with *F. graminearum;* disease is radiating out from one spot. Fig. 24. Five nematodes and two eggs in a small root lesion. Fig. 25. Numerous bacteria growing in a nematode tunnel.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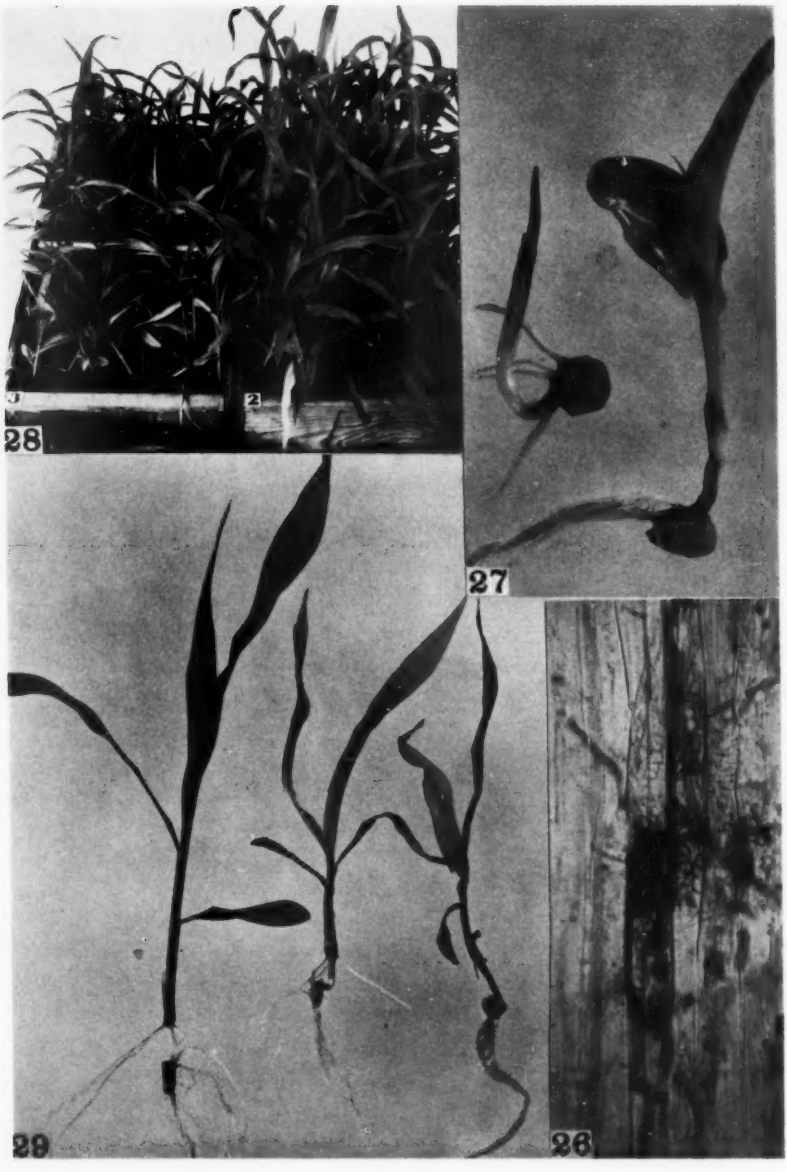
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Fusarium graminearum BLIGHT

This fungus has frequently been considered the most important ear and root rot organism of corn. From these studies, however, its role as a true root rot organism appears to be minor. In fact, only one isolate was obtained from diseased roots in more than several hundred attempts made from seedlings grown in several "root rot" soils. The single isolate obtained was much more virulent than some which were obtained from diseased ears, and this finding agrees with Ullstrup's work (14) on the variability of pathogenicity of *Gibberella saubinetii*.

The incidence and severity of seedling blight produced by *F. graminearum* increased as the temperature was lowered, but, under such conditions, the interval prior to the first symptom of disease became progressively longer. The disease frequently radiated outwards from a certain locus in the flat (Fig. 23). Root lesions as well as mesocotyl necrosis were quite noticeable. Inbred A was more susceptible than W26 to both the *Fusarium* species studied.

TABLE III

EFFECT OF TEMPERATURE ON THE INCIDENCE OF SEEDLING BLIGHT IN STERILE SOIL INOCULATED WITH *F. graminearum*

Temperature	First appearance of blight	Percentage		
		Emergence	Postemergence blight	Final stand
24	—	90.0	0.0	90.0
20	14*	96.3	75.3	23.8
16	23	95.0	91.9	7.5
12	32	68.8	63.6	13.8

* *Days after planting.*

Rhizoctonia solani (DAMPING-OFF)

This organism proved to be widely distributed but of very little economic significance. Microscopic observations have shown that it frequently grows adjacent to the roots and occasionally enters some of the outer epidermal cells and completely fills them with its segmented mycelium (Fig. 26). On the corn roots, light to dark brown dry lesions may be quite numerous, but they are always superficial and localized, and cause only slight stunting.

FIG. 26. *R. solani* mycelial segments filling some of epidermal cells of a corn root. FIG. 27. The symptoms on very young corn seedlings after one has been infected in the growing tip and the other in the primary leaf; the roots are healthy. FIG. 28. No. 2 is a check flat; No. 3 was inoculated with *R. solani*. In the latter, many of the seedlings have been attacked at the ground level. FIG. 29. Seedlings showing symptoms due to *R. solani* infection. From left to right: healthy; seedling infected above the crown; later stage, but observe that the root is healthy.

This fungus, however, has been observed to attack vigorously the coleoptile and young leaves (Fig. 27), and the plant dies immediately if infection spreads into the growing point. Sometimes infection remains localized in the first or second leaf and, under such circumstances, they frequently do not open but remain wrapped around the inner leaves, with the result that the plant resembles one suffering from the genetic disorder known as "rolling".

Disease incidence was quite severe at 20° C., but was not serious at the other temperatures used (Table IV). The seedlings were killed even after they were several inches high (Figs. 28 and 29), and it will be noticed that infection occurred at the soil surface, as is so common with this organism. The roots remained healthy.

TABLE IV

EFFECT OF TEMPERATURE ON THE INCIDENCE OF SEEDLING BLIGHT IN STERILE SOIL INOCULATED WITH *R. solani*

Temperature	Percentage		
	Emergence	Postemergence blight	Final stand
24	82.5	4.5	78.8
20	90.0	75.0	20.0
16	92.5	5.4	87.5
12	87.5	0.0	87.5

NEMATODES

Microscopic observations have shown that, under natural conditions, nematodes are always present in the lesions produced by *Pythium*, and frequently several may be present in one small lesion (Fig. 24). Apparently they are able to complete much of their life cycle within the root tissue, because eggs, larvae, and adults are found among the *Pythium* oogonia. It would appear that the nematodes drag many organisms in and out of the roots (Fig. 25), and cause considerable wounding and tunnelling. A surprisingly large number of nematodes were found in young diseased roots of corn seedlings which were grown in steam-sterilized soil that was inoculated with *P. arrhenomanes* and contained in clean formalin-treated flats.

The counts indicated in Table V include all nematode species present, but no attempt was made to identify them. This experiment proves that a considerable number of nematodes may invade healthy root tissue or perhaps live in their rhizosphere. If the nematodes invaded healthy root tissue, they must have been responsible for numerous injuries, but such plants appeared to be quite as healthy as those containing practically no nematodes, and thus did not appear to contribute to the damage caused by *P. arrhenomanes*. Nematodes were almost absent in the steamed flats, but were very numerous in the nonsteamed flats and became more abundant after 29 days. It is apparent that nematodes cling to flats and are not all destroyed when the flats are immersed in a 4% formalin solution for two minutes.

TABLE V

THE EFFECT OF STEAMING SOIL FLATS ON THE ABUNDANCE OF NEMATODES IN HEALTHY AND IN INOCULATED ROOTS OF CORN SEEDLINGS

Treatment	No. of nematodes per gm. of root tissue	
	13 Days after planting	29 Days after planting
Steamed flat, noninoculated soil	0.0	0 0
Steamed flat, inoculated soil	1.2	2.5
Nonsteamed flat,* uninoculated soil	0.7	40.9
Nonsteamed flat,* inoculated soil	12.7	127.1

* *Flats were immersed in 4% formalin solution.*

Discussion

Of the several root rot pathogens of corn that were isolated and studied in southern Ontario, *P. arrhenomanes* was the most important individual component of the complex and was present throughout the whole corn-growing area. This finding is in agreement with Valleau *et al.* (16), who state that, "The suggestion is made that corn rootrot, other than seedling blights known to be caused by certain seed-borne organisms, is caused by a fungus similar to the fungus causing the cane rootrot".

Even though *F. moniliforme* was found in most necrotic roots, this organism is apparently of only minor importance, since it attacks only after ideal environmental conditions have been maintained for several weeks, a circumstance which seldom occurs under natural conditions. The fact that the seedlings usually survive also substantiates this belief. Johann, Holbert, and Dickson (7) have indicated also that this organism is of only secondary importance. Leonian (10) believes that *F. moniliforme* produces few, if any, positive infections through sound tissues, and he states that "this assumption is particularly strengthened by the fact that when, in a series of experiments, the soil was sterilized, heavily inoculated with rice cultures of *F. moniliforme* and planted to corn, no seedling diseases could be observed".

Some investigators have found that a wheat–corn rotation is favorable to the survival and development of *F. graminearum*, but, in this investigation, *F. graminearum* was practically absent and apparently of no importance. Dickson (1) found that this organism caused heavy losses, especially when corn and wheat are in close sequence in the crop rotation. In Iowa, Wen-Chun Ho (5) states that *Gibberella saubinetii* was the organism most commonly isolated from the necrotic lesions on roots and mesocotyls of maize seedlings.

This work has shown that reduction in size and vigor of seedling corn plants is characteristic of *Pythium* injury under field conditions but that reduced germination and postemergence dying seldom occurs. The injured plants frequently show marked recovery, but, since the potentialities of the plants are determined during the first part of the growing season, injury is expressed in yield reduction and sometimes no grain develops on the dwarfed plants.

All evidence indicates that the disease under investigation is favored by high soil moisture, and this is readily understandable since free water is necessary for motility of the zoospores. Although aggressive parasitism occurs throughout a wide temperature range, providing other conditions are favorable, the severity of the disease increases with decrease in temperature. On the other hand, *Pythium* root rot of wheat, caused by the same organism, is most severe at relatively high temperatures. In this case it appears that the optimum temperature for the disease is determined by the particular host plant that the fungus attacks. Corn, whose optimum temperature is about 30° C., is more likely to be adversely affected by lower temperatures and, therefore, becomes more susceptible at these temperatures, whereas, with wheat, the converse is true. An analogous instance has already been reported by Dickson *et al.* (1) for *Gibberella saubinetii* (Mont) Sacc. attacking corn and wheat.

In the writer's experiments, Arasan seed treatment only ensured seed protection, but Hoppe (6) found that Arasan seed treatment afforded excellent control against stunting due to *Pythium* in both naturally infected and artificially inoculated soils. Apparently Hoppe was using a different species or a less virulent strain, because dwarfing was not prevented in any of the present experiments.

Although nematodes were found in almost all necrotic lesions produced by *Pythium* and became more abundant as *Pythium* root rot increased, they appeared to be of little significance in the disease complex. Graham and Holdeman (3), however, have recently reported that *Pratylenchus* alone causes much root rot of corn and that population counts decreased rapidly before maximum root decay occurred. Apparently the writer was not dealing with this species, because true nematode decay was not observed. It was found, however, that these organisms survived on formalin-treated flats and thus readily gained entrance into the steam-sterilized soil.

Since the most important pathogen of this seedling disease is a very unspecialized parasite, it is most difficult to obtain a highly resistant or immune plant, although such would be the most effective and least expensive method of control. Soil fungicides are available which will destroy this organism, but their high cost at the present time militates against their practical use. Probably other chemical and cultural practices which partially control this disease will have to be used in the immediate future.

Acknowledgments

I am deeply indebted to Dr. L. W. Koch for his advice during this investigation and criticism of the paper. Acknowledgment is due G. C. Mortimore, of the Dominion Experimental Station, Harrow, for supplying all seed used and especially to W. B. Mountain, of the Harrow laboratory, for nematode counts.

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A COMPARISON OF SEED TESTING METHODS IN RELATION TO THE STEM-BREAK AND BROWNING DISEASE OF FLAX, CAUSED BY *POLYSPORA LINI* LAFF.[1]

By V. R. Wallen and A. J. Skolko[2]

Abstract

A comparison of the Ulster, New Zealand, and Ottawa methods of seed examination for the presence of *Polyspora lini* Laff., the cause of stem-break and browning disease of flax, showed great variation in the percentage of infection obtained, depending upon the method used. The Ulster method, in which the seed is not treated before it is plated, does not differentiate deep-seated infection from superficial infection of the seed, but has proved satisfactory in actual practice with seed samples that are not contaminated seriously by saprophytic fungi. The New Zealand method, by which surface contamination is supposedly removed by washing the seed in running water, has not been found reliable. The Ottawa method, by which the seed is surface sterilized before it is plated, does not indicate the degree of superficial infection, but does give the percentage of deep-seated infection, and hence it indicates the suitability of the seed for planting purposes if the seed is treated with a fungicide. It is suggested that a centrifuge test combined with the Ottawa plating method would provide a better index of the health condition of flax seed as far as disease caused by *P. lini* is concerned.

Introduction

Standardization of seed testing methods in respect to germination and purity has been attained through the Association of Official Seed Analysts of North America and, at the international level, through the International Seed Testing Association. Much less agreement exists in the matter of testing seed for the presence of pathogenic organisms. The reason for this lack of uniformity in methods of disease diagnosis may be attributed primarily to the development by individual laboratories of seed testing methods especially suitable to their own requirements, facilities, and personnel. Standardization has undoubtedly also been hampered by the fact that comparatively little is known of the seed-borne phases of many of these organisms, the failure to develop satisfactory methods of detection for seed-borne pathogens, and the emphasis on the empirical application of seed treating procedures for their control, with the consequent neglect of fundamental information as to how the seed treatment is operative. As a consequence of the differences in seed testing methods, misunderstandings are inevitable and proper interpretation of reports is not possible.

By way of illustrating how such misunderstandings can result from the use of different methods of testing, mention may be made of a particular shipment of flax seed intended for export to Canada. A sample of the seed was tested for seed-borne fungi according to the method in use at the Ottawa laboratory. It was found to be internally infected to the extent of 1% of the seed with

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Contribution No. 1064 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

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Polyspora lini Laff. and externally infested with the same fungus to the extent of 48%. This information was transmitted to the prospective exporters who were at a loss to understand these results in view of their own examination which showed between 7 and 14% infection with *P. lini*. Subsequent inquiry on their part disclosed that the two reports were based upon different testing methods. As an outcome of this situation, it was decided to make a comparison of the several methods of testing for the presence of *P. lini* which have been described. The results of these tests are given below.

The disease, known as stem-break and browning of flax and caused by *Polyspora lini* Lafferty, may kill young seed in the capsule (2) or may produce a brown spotting of the cotyledons, stem, leaves, petioles, and seed capsules. The brown spots often coalesce to form elongated lesions, which give to infected plants a brown appearance. Lesions on the stem weaken the plant and may result in a bending or breaking of the stem, the so-called "stem-break" symptom. The disease is of world-wide distribution but of variable importance.

Experimental

Three methods of seed testing for the presence of *P. lini* were compared. For present purposes they may be designated as the Ulster, the New Zealand, and the Ottawa method, and are briefly described, as follows:

Ulster Method

"The seed sample to be examined is thoroughly mixed and the seeds plated out on 2% malt extract agar poured into Petri dishes (9.0 cm. diam.); ten seeds are spaced equidistantly in each dish. . . . . Forceps are used for the transfer of each seed to the medium, and the points of these are sterilized by dipping in alcohol and flaming after the plating out of each lot of ten seeds. The dishes are then incubated at 22° C., and after 5 days the medium around each seed is examined for the growth of parasitic organisms" (3).

New Zealand Method

"The seed sample is thoroughly mixed. About one thousand seeds are placed in a close-mesh wire-gauze cage and vigorously washed for five minutes with fast-running tap water, followed by a final rinse in sterile water. This removes most of the surface-borne spores and mycelium. The water is drained off, and forty to fifty of the wet, mucilaginous seeds are dispersed on the bottom of 9-cm.-diameter Petri dishes and are then covered by nutrient agar at 45° C. The dishes are incubated for five to seven days at 22° C." (4).

Ottawa Method

"Samples of 100 seeds were tied in cheesecloth bags and surface sterilized by immersing them in a solution made from commercial chloride of lime, standardized to 2% available chlorine. The time of immersion varied from 2 to 20 min. according to the kind of seed. (Ten min. for flax seed). . . . . .

The seeds were then placed by means of flamed forceps directly on 2% malt extract agar in Petri dishes (5 seeds per dish) which were stored at room temperature. Readings were taken after 10 days" (1).

The essential difference between the three methods may be summarized as follows:

(*a*) *Ulster*—no surface sterilization of the seed; seeds plated on the surface of the agar.

(*b*) *New Zealand*—surface of the seeds washed in running tap water; seeds plated and then covered with the agar medium.

(*c*) *Ottawa*—seeds surface sterilized with 2% chlorine solution; seeds plated on the surface of the agar.

Representative plates illustrating the three methods are shown in Fig. 1.

In order to determine accurately the percentage of the seeds carrying surface-borne *P. lini*, and thus clarify differences in the methods, a centrifuge test was made although this step is not normally included in the Ottawa method. In the centrifuge test, seeds were placed separately in individual test tubes in 2 ml. of sterile water. The tubes were shaken 30 times, allowed to stand for 18 hr., and then centrifuged. The precipitate was then examined microscopically for the presence of spores of *P. lini*.

Two lots of flax seed, known to be infected with *Polyspora lini*, were used in making the comparative tests of the three methods. One lot was of the variety Royal, No. 205, grown at Marshall, Sask.; the other was the variety Stormont Motley, No. 571, received from Belfast, Northern Ireland. The results of the comparative test and the centrifuge test are presented in Table I.

TABLE I

PERCENTAGE OF SEEDS INFECTED WITH *Polyspora lini* AS DETERMINED BY THE ULSTER, THE NEW ZEALAND, AND THE OTTAWA METHOD

Sample No.	Method	No. of seeds	*Polyspora lini*, %
205	Ulster	1000	9.2
205	New Zealand	1000	21.7
205	Ottawa (plate)	1000	2.6
205	" (centrifuge)	500	24.3
571	Ulster	300*	13.0
571	New Zealand	200	54.0
571	Ottawa (plate)	300	3.0
571	" (centrifuge)	100	48.0

* *Smaller samples made necessary because of limited quantity of seed.*

It is apparent from Table I that great variation in the percentage of *Polyspora lini* may be obtained, depending upon the method used. In sample No. 571, the percentage of *P. lini* ranged from 3.0 to 13.0 and 54.0 and in

PLATE I

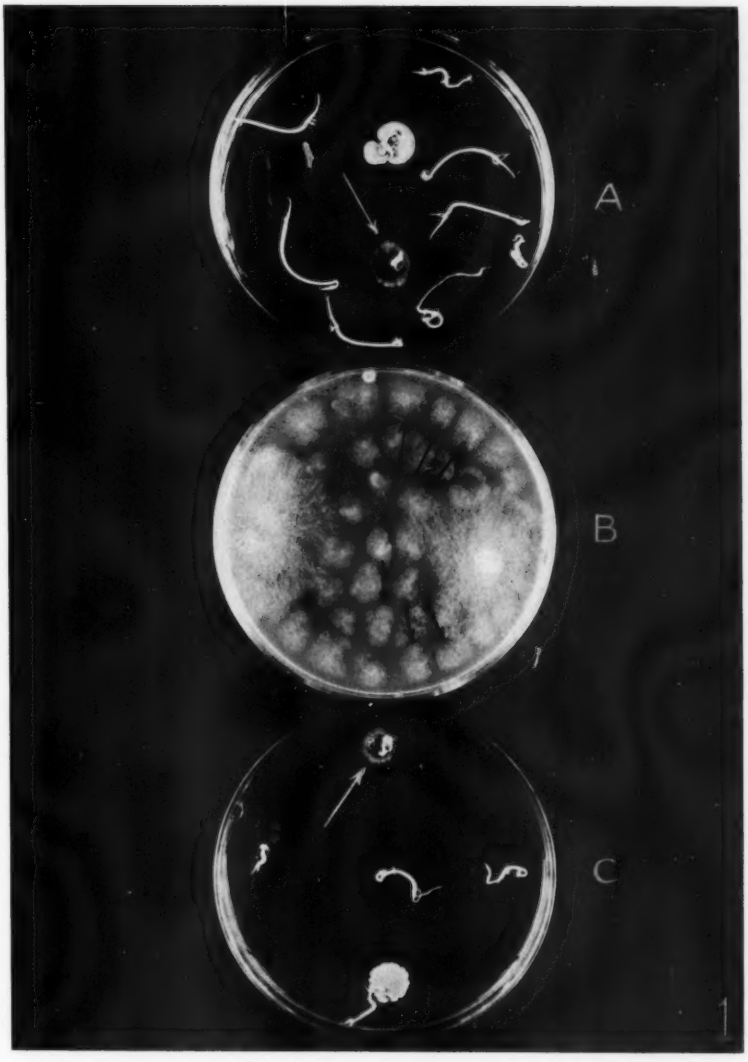

FIG. 1. Flax seed, sample No. 205, plated according to A. Ulster method; B. New Zealand method; and C. Ottawa method. Arrows indicate *Polyspora lini* colonies.

sample No. 205, from 2.6 to 9.2 and 21.7 for the Ottawa, the Ulster, and the New Zealand method, respectively. The differences between the three methods readily account for the variation in the results obtained. The low percentage of *P. lini* obtained in both samples by the Ottawa plate method (2.6 and 3.0%) as compared with the Ulster method (9.2 and 13.0%) is to be expected because, by the Ottawa method, the seed is first sterilized. The high percentage of *P. lini* as determined by the New Zealand method (21.7 and 54.0) is difficult to understand as it is claimed by Newhook (4) that washing the seed in running tap water removes surface spores and mycelium and gives a better estimate of internal infection with *P. lini*. Therefore, the Ulster method should give a higher percentage of *P. lini* than should the New Zealand method, as, in the former method, no attempt is made to remove surface-borne *P. lini*. In actual tests carried out by Newhook (4) the Ulster method gave only 15% as compared with over 26% by the New Zealand method. This apparent contradiction was attributed by Newhook to the presence of saprophytes which suppress and mask the growth of *P. lini*. Furthermore, Newhook claims that with the Ulster method only one side of the seed is in contact with the medium and germination of the seed lifts the seed coat above the agar surface, whereas, with the New Zealand method, the seeds are submerged in the agar. In the present tests, however, the first objection to the Ulster method is hardly tenable, as very few saprophytes were present, especially in the sample of Northern Ireland origin. It may also be mentioned that the New Zealand method provides no data on the germinative capacity of the seed.

A comparison of the percentages of *P. lini* obtained by the combined Ottawa plate and centrifuge tests on one hand and the New Zealand method on the other, shows fairly close agreement (26.9 and 21.7%, respectively, in one sample and 51.0 and 54.0%, in the other). The inference to be derived from these figures is that, in effect, surface-borne *P. lini* is not removed by washing in running tap water. It is even conceivable that, in running water, surface spores and mycelium washed from infected seeds may contaminate uninfected seeds and that the mucilaginous nature of the seed coat favors this contamination.

In view of Lafferty's (2) histological studies in which he found that, in infected seed, the mycelium develops in the outer layers of the seed coat only, it is perhaps academic to argue whether the seed is internally infected or whether the infection is externally borne. It is of importance, however, to know whether infection is superficial enough to be controllable by chemical seed treatment or whether it is deep-seated and hence not affected by such treatment. Of the three methods, the Ottawa test, whereby the seed is surface sterilized before plating, gives some indication whether the seed is suitable for seeding purposes if the seed is treated. It does not indicate how much surface-borne *P. lini* is present, and hence does not indicate whether or not seed treatment should be recommended. However, a centrifuge test of a bulk sample of seed to supplement the plate test would provide a basis for

such a recommendation. By plating the precipitate from the centrifuged sample, the viability and identity of *P. lini* could be verified. Possibly by combining certain features of the New Zealand and Ottawa methods, a method could be devised that eliminates the undesirable features of both. For instance, surface disinfection according to the Ottawa method and plating the seed submerged in the agar would make evident the existence of deep-seated infection; and an examination of the centrifuged washings would show whether or not the seed is superficially infected, and hence, whether or not seed treatment is necessary.

It is not the purpose of this paper to suggest which of the three methods should be adopted in seed testing procedure but rather to indicate the necessity of defining the method on which a given report is based. In actual practice the Ulster method has proved quite satisfactory from the standpoint of rapidity, and of reliability as substantiated by field trials (3). It is perhaps best suited to the examination of flax seed grown in Northern Ireland and Great Britain, where contaminating saprophytes are much less general than in Canadian-grown flax seed.

Acknowledgment

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THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

V. THE IDENTIFICATION OF PYRUVATE AS AN INTERMEDIATE IN GLUCOSE OXIDATION[1]

By Roger H. Warburton,[2] B. A. Eagles,[3] and J. J. R. Campbell[4]

Abstract

Pyruvate has been determined at 16, 28, and 40 hr. in a culture of *Pseudomonas aeruginosa* when growing in a glucose medium. Throughout this interval the organism was shown to possess the enzyme system capable of rapidly oxidizing pyruvate. It was concluded that pyruvate is being formed and dissimilated continuously and that it is therefore to be considered an intermediate in the oxidation of glucose. It was found that glucose oxidation was not inhibited by 0.02 *M* fluoride and that pyruvate formation and utilization continued unimpaired in the presence of the inhibitor. This would indicate that enolase is not essential to the formation of pyruvate by *P. aeruginosa* and may be taken as further evidence supporting the contention that this organism does not dissimilate glucose by way of the conventional Embden–Meyerhof scheme.

Recent work from this laboratory has demonstrated that *Pseudomonas aeruginosa* 9027 oxidizes glucose by a pathway which differs from any previously described for tissue or microorganisms. It has been established that glucose is oxidized to gluconic acid which in turn is oxidized to 2-ketogluconic (6, 7). It has also been shown that acetate is an intermediate in the oxidation of glucose (3). The major importance of this pathway to *P. aeruginosa* was confirmed when it was found that none of the members of the Embden–Meyerhof scheme could be detected as intermediates (2).

The present study was undertaken to determine whether or not pyruvate is an intermediate in the breakdown of glucose by *P. aeruginosa* 9027.

Methods

Pseudomonas aeruginosa A.T.C. 9027 was used as the test organism. The cultural methods and media employed were the same as recorded earlier (6).

The conventional Warburg apparatus was employed for the respiration studies. Cells were harvested at periods of 16, 28, and 40 hr., standardized using the Fisher Electrophotometer, and the same concentration used in all Warburg vessels.

For detecting the formation of pyruvate during growth, determinations were carried out on the growth medium after the removal of the cells by centrifugation, adjustment to pH 7.0, and concentration *in vacuo* at 39° C.,

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Contribution from the Department of Dairying, The University of British Columbia, Vancouver, B.C. This study was carried out under a grant from the National Research Council of Canada, laboratory facilities being found by the University of British Columbia.

[2] *Graduate Student.*

[3] *Professor of Dairying.*

[4] *Associate Professor of Dairying.*

to 1/10th of its original volume. In order to ensure a protein concentration equivalent to that present in the experimental flasks the addition of harvested cells to the uninoculated control immediately prior to the determination of pyruvate was carried out. False positive tests for pyruvate attributable to the degradation of cellular material remaining after centrifugation were thus avoided.

The technique of paper chromatography was employed for the detection of pyruvate using the 2,4-dinitrophenylhydrazone procedure of Cavallini, Frontali, and Toschi (4). Whatman No. 1 filter paper was used and the ascending chromatographs were irrigated for a period of 17 hr. at room temperature. Confirmation of the presence of pyruvate in the concentrate was obtained by Procedure B of Friedemann and Haugen (5) using toluene as the solvent and Filter No. 525 for the electrophotometer readings.

Experimental and Discussion

The analysis of the glucose medium after *P. aeruginosa* had been growing for 16, 28, and 40 hr. revealed that pyruvate was present over the entire 24 hr. interval (Table I). It was also found that cells harvested at these same

TABLE I

R_f VALUES OF THE 2,4-DINITROPHENYLHYDRAZONES FORMED FROM THE GROWTH MEDIA

Treatment	Compounds	Solvents*		
		1	2	3
2,4-Dinitrophenylhydrazones of reference substances	*Oxalacetate*	0.43 .63	0.17 .47	0.14** .47
	α-Ketoglutarate	.51	.11	.16
	Pyruvate	.61	.45	.46
2,4-Dinitrophenylhydrazones formed with glucose growth medium	*At 16 hr.*	.62	.46	.47
	At 28 hr.	.61	.45	.47
	At 40 hr.	.61	.46	.48
2,4-Dinitrophenylhydrazones formed with glucose growth medium containing fluoride	*At 16 hr.*	.63	.46	.46
	At 28 hr.	.61	.46	.48
	At 40 hr.	.62	.46	.48

* *1. n-Butanol saturated with 3% acetic acid.*
2. n-Butanol saturated with 3% ammonium hydroxide.
3. n-Butanol 50%, ethanol 10%, and distilled water 40%.
** *Two R_f values are recorded for oxalacetate indicating partial breakdown to pyruvate.*

periods were capable of oxidizing pyruvate immediately when tested in the Warburg respirometer (Fig. 1). Since pyruvic acid is detectable in the glucose growth medium for a 24 hr. period (16-40) hr.) (Table I) and since the organism has the enzyme system necessary for the oxidation of this acid (Fig. 1), it is to be concluded that throughout the 24 hr. interval pyruvate is being formed and oxidized continuously and is therefore an intermediate in the oxidation of glucose.

The demonstration that pyruvic acid is an intermediate in the oxidation of glucose by *P. aeruginosa* might be interpreted as evidence supporting the presence of an Embden–Meyerhof system. In agreement with results

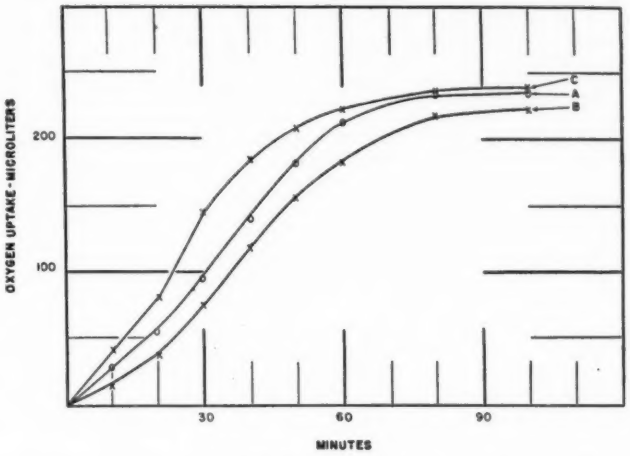

FIG. 1. Pyruvate oxidation by cells grown on glucose medium. Warburg cup contained 0.5 ml. of cell suspension; 1.5 ml. *M*/15 phosphate buffer pH 7.2; 0.2 ml. of substrate; 0.15 ml. 20% potassium hydroxide. Final volume was 3.0 ml. Theoretical oxygen uptake for complete oxidation was 403 microliters. Curves A, B, C, – pyruvate oxidation by 16 hr. cells, 28 hr. cells, and 40 hr. cells respectively.

reported by Barron and Friedemann (1) it was found that the dissimilation of glucose by this organism was not inhibited by 0.02 *M* sodium fluoride. It was also found that when the organism was grown in a glucose medium to which fluoride had been added to a concentration of 0.02 *M* pyruvate could be detected at 16, 28, and 40 hr. Cells harvested from these fluoride containing media were found to have an unimpaired ability to oxidize glucose or pyruvate. From the data obtained it would appear that the enzyme enolase is not essential to the formation of pyruvate by *P. aeruginosa* and this finding may be taken as further evidence supporting the contention that this organism does not dissimilate glucose by way of the Embden–Meyerhof scheme.

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NOTES ON THE CANADIAN TUCKAHOE, ITS OCCURRENCE IN CANADA AND THE INTERFERTILITY OF ITS PERFECT STAGE, *POLYPORUS TUBERASTER* JACQ. EX FRIES[1]

By T. C. Vanterpool[2] and Ruth Macrae[3]

Abstract

The Canadian tuckahoe is the perennial sclerotium of *Polyporus tuberaster* Jacq. ex Fries. It is commonly found in the parkland belt of the Canadian prairies where land supporting virgin poplar groves, mainly *Populus tremuloides* Michx., is being brought under cultivation. Sporophores, as many as three to a single sclerotium, appear in late June and July. Interfertility studies with single spore cultures isolated from sporophores derived from four sources in Western Canada and from one source in Italy have shown that both the Canadian fungus and the European *P. tuberaster* are heterothallic, have the tetrapolar type of interfertility, and are interfertile.

The Canadian tuckahoe is a large, black sclerotium, which is the resting stage of the fungus *Polyporus tuberaster* Jacq. ex Fries (Figs. 1 to 9). The tuckahoe is found in the black soil and occasionally in the transition soil zones of the Canadian prairies extending from southeastern Manitoba, northwest through Saskatchewan and Alberta, into British Columbia. The black soil zone or parkland prairie is the aspen-grove belt, and adjoining it to the north is the transition zone consisting of mixed black, degraded black, and gray soils, and a parkland–forest vegetation. The groves in which the sclerotia are found are invariably on light-textured soil. In Manitoba, collections have been made as far south as the Pembina Hills, and it is suspected that the range of the fungus extends into North Dakota (2). In Saskatchewan, all known collections are from the belt parallel to and northeast of a rough line running from Moosomin in the southeast, through Touchwood and Radisson, to Senlac in the northwest of the province. The most westerly collections are from Bridge Lake, 70 miles north of Kamloops, B.C., at an elevation of 3650 ft.

The localities in Canada where sclerotia have been found to date are given here, along with the source from which each of the records was obtained. *Manitoba:* Cromwell, Gilbert Plains, Holland, Mekiwin, Miami, Moose Nose, and Notre Dame de Lourdes by Fletcher (5); Carmen, Clear Lake, Kenville, Oakville, near the Pembina Hills, and Swan River by Bisby *et al.* (2); Raeburn in the Mycological Herbarium, Department of Agriculture, Ottawa (DAOM F2345). *Saskatchewan:* Duck Lake, Moosomin, St. Louis, Togo, and Welwyn

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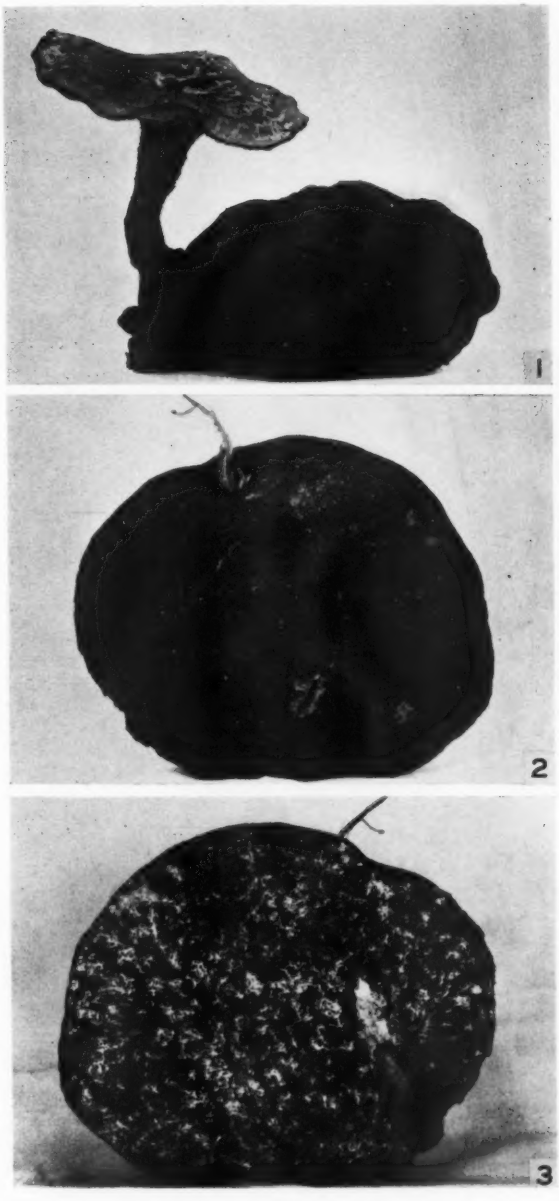
by Fletcher (5); Caragana and Watson in the Herbarium, Dominion Laboratory of Plant Pathology, Saskatoon; Chelan, Delmas, Megan, Plunkett, Pontrilas, and Senlac from unpublished records of W. P. Fraser, formerly Professor of Biology, University of Saskatchewan; Archerville, Beatty, Border Road, Cleeves, Churchbridge, Englefield, Fairy Glen, Goodsoil, Kilwinning, Melfort, Paddockwood, Petaigan, Radisson, Red Jacket, St. Brieux, Silton, South Makwa, Steen, and Touchwood from the records of the senior author. *Alberta:* Fort Saskatchewan reported by E. H. Moss, Professor of Botany, University of Alberta; Grande Prairie (DAOM F984). *British Columbia:* Bridge Lake reported by P. E. M. Leith.

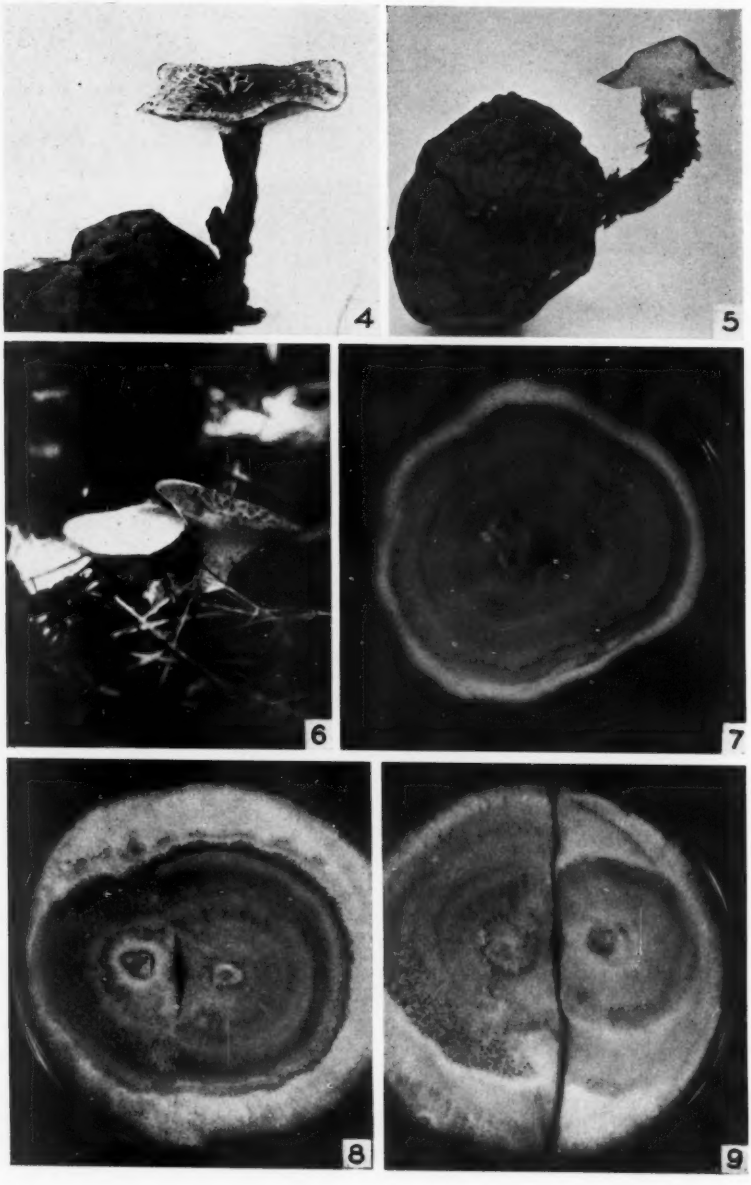
A preliminary account of the sclerotia was given in 1907 by Fletcher (5), who showed that they were the resting bodies of a species of *Polyporus*. In 1919, Güssow (6) reported that he had obtained a sporophore from a sclerotium buried in earth in a flower pot about 10 months previously. He gave detailed descriptions of the sclerotia of the fungus and of the sporophore to which little of significance may be added here. The illustrations in the present paper (Figs. 1 to 6) will serve as additional visual aids to his descriptions.

About two or three tuckahoe bodies are received at the Department of Biology, University of Saskatchewan, every year from the parkland belt where virgin land covered with poplar groves is being cleared and brought under the plough. The Geology Department, the History Museum, and the Chemistry Department of the University also receive occasional specimens. Correspondents invariably mention the association with poplars, the aspen (*Populus tremuloides* Michx.) being the only one specifically mentioned. The undisturbed bodies are found with their tops about two to six inches under the surface of the soil. In one instance a group of approximately fifty was dug up from a small area, but this number is considered exceptional. Sometimes small poplar roots are found growing through a specimen (Figs. 2 and 3). These facts suggest an intimate relationship between the fungus and the poplar root, but no mycorrhizal or parasitic relationship has yet been established. Rarely, willow (*Salix*) is mentioned as being present among the poplars, but whether or not this association is merely accidental needs elucidation. Sclerotia exposed by the plough soon dry out and usually fail to produce sporophores when they are buried in moist soil. Bisby *et al.* (2) report an instance of a fruit body being found attached to its sclerotium six inches below ground level in a field four years after the land was first ploughed. In April this year Dr. E. H. Moss of the University of Alberta sent us a photograph of a young sclerotium showing portions of two rhizomorphs about 2 to 3 mm.

Polyporus tuberaster. FIG. 1. A mature sporophore which developed in the middle of July, 1936, from the sclerotium buried on October 21, 1935. $\frac{1}{3}$ × natural size. FIG. 2. Surface view of a sclerotium; a shredded portion of a poplar root is seen emerging at the top. $\frac{2}{3}$ × natural size. FIG. 3. Internal view of the sclerotium shown in Fig. 2, showing light patches of hyphae with medium-thick walls and clamp connections, and dark patches of extremely thick-walled hyphae; a diagonal section of the poplar root is seen at the lower right. $\frac{2}{3}$ × natural size.

PLATE 1

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thick and 2 cm. long still attached to opposite ends of the longitudinal axis. He describes the specimen as being quite soft, light colored, and approximately 5.5 × 4.0 × 1.5 cm. in size. It was sent to him by Dr. G. H. Turner from Fort Saskatchewan, Alta., on June 28, 1940, where it was found on newly broken land. To our knowledge this is the only specimen in which rhizomorphs have been observed.

The sclerotia are spherical to oval and occasionally slightly flattened. They are of a hard, rubbery consistency when fresh, but dry out to the hardness of a stone. They have a great capacity for absorbing water; one dry specimen weighing 4.14 lb. absorbed 47.2% water, based on its air-dry weight. They vary in size from about 2 to 8 in. in diameter, and the largest may weigh between 8 and 9 lb. The largest specimen reported to us measured 16 in. × 10 in. × 4 in., but its weight was not given. They are sometimes referred to as 'Indian bread' or are wrongly mistaken for pemmican or dried buffalo meat of the Indians, but even the softest are much too tough to be eaten and do not soften appreciably when boiled. However, they do appear to have been used by the Indians for poulticing and for rheumatism and were called "ground medicine" by them. The top of one pileus, which developed in 1936 from a sclerotium buried under poplars at Saskatoon in 1935 and is now in the herbarium of the University, appears to have been pecked by birds. The pilei vary from 2 to 6 in. in diameter, and the sporophores from 2 to 5 in. in height. As many as three sporophores may develop from a single sclerotium at the same time (Fig. 6).

Cultures of the fungus are readily obtained from internal sclerotial tissue and from basidiospores. The mycelium is white at first, later changing to pinkish buff, tan-buff, and then to brown. The colored areas and zones are usually restricted to the region around the inoculum. The margin of a culture is usually white (Figs. 7 to 9). Dark lines sometimes develop in old flask cultures, but no gristly sclerotial tissue and black rind in any way resembling the natural body are formed. Sporophores develop fairly readily after about five weeks in flask cultures, but they are rarely well formed. For detailed growth and mycelial characters reference should be made to the full description given by Nobles (10).

Over a period of many years, several perfect sporophores have been obtained by burying fresh sclerotia from one to three inches below ground level either directly in poplar groves or in pots of soil buried level with the ground. The fruiting period extends from about the last week of June to the end of July, the majority of the sporophores emerging during early July and lasting for

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Polyporus tuberaster. FIGS. 4 and 5. Sporophores and sclerotia. ⅓ × natural size. FIG. 6. Two sporophores which developed in the middle of July, 1947, from a sclerotium buried on May 29 the same year. ⅓ × natural size. FIG. 7. Mass spore culture on potato dextrose agar 24 days old. ⅔ × natural size. FIG. 8. Two monocaryon cultures of compatible interfertility groups growing together and forming clamp connections. ⅔ × natural size. FIG. 9. Two monocaryon cultures showing the frequent nonintermingling of strains of incompatible interfertility groups. ⅔ × natural size.

from 10 days to two weeks. Ordinarily, specimens received during the summer months and buried immediately do not fruit until the following season. One fresh specimen received on May 29, 1947, was buried the same day 2 in. deep and 18 in. from the base of a poplar sapling in a grove on the University grounds. Two sporophores appeared above the surface of the ground (Fig. 6) during the first week of July of the same year, and by July 11 were sporulating freely. The sclerotium was left undisturbed and in July, 1948, rudiments of fruit bodies were observed; but three days later they were found to have been broken off or otherwise injured, and no further sporophore development occurred during 1948. On July 6, 1949, another developing sporophore was observed. This one attained full development and finally collapsed on July 23. It would naturally be suspected that a storage organ as large as a tuckahoe would serve as a perennial structure, giving rise to fresh sporophores year after year. Bisby *et al.* (2) have reported a case in which a sclerotium buried in damp earth in a basement produced sporophores three years in succession, but no reports were received of the development of sporophores in subsequent years.

Taxonomy and Nomenclature

The name 'tuckahoe' was first given to the sclerotium-producing fungus of Western Canada by Fletcher (5) in a preliminary report on the fungus entitled *A Northwest Tuckahoe* published in 1907. The name was suggested to him by the fact that Dr. Farlow considered the fungus to be most nearly allied with the Southern tuckahoe or 'Indian Bread' of the Southeastern United States. Güssow (6), in his paper published in 1919, used the name "Canadian tuckahoe" for the fungus, although he believed it was not the same as the southern species. Weber (14), in 1929, found that the fruiting stage of the Southern tuckahoe is *Poria Cocos* (Schw.) Wolf, thus proving that the sclerotia found in the south belong to a resupinate fungus quite different from the stipitate species of Western Canada.

The first record of the fruiting stage of the Canadian fungus was made by Fletcher (5) in 1907. He gave a very brief description of two sporophores which had grown from a sclerotium he had planted and referred them to the genus *Polyporus* but did not identify them further. Fletcher had sent sclerotia to Dr. Farlow and one of them, which the latter had given to Dr. Roland Thaxter, also fruited. Thaxter wrote Fletcher (5) concerning it: "I sent the Polyporus directly to Dr. Farlow who recognized it as a European species which I believe he had not seen before." Güssow (6) also obtained a sporophore from a sclerotium from Western Canada which he had planted, and, in 1919, he described the fungus as a new species, naming it *Grifola Tuckahoe*. Lloyd (7), in 1920, referring to Güssow's paper, said that he had examined a sclerotium of the Canadian fungus and that, on comparison with the sclerotium of *Polyporus tuberaster* of Italy, it seemed to be the same. He believed that when better known the Canadian fungus would be found to be that species. *P. tuberaster* is a species found in Europe and long known from

Italy, where the fruit bodies have been cultivated for food. In 1925, Saccardo and Trotter (12) placed the Canadian fungus in the genus *Polyporus*, calling it *Polyporus Tuckahoe* (Güssow) Sacc. et Trott., and Güssow, in 1929, concurred with this concept (Mounce (8)). Davidson *et al.* (4), in 1938, and Nobles (10), in 1948, used the binomial *Polyporus tuberaster* for the fungus. The latter author states that cultures received from Europe appear to be identical with those obtained from sclerotia or fruit bodies originating in Western Canada. The results of interfertility studies with the fungus from Western Canada and from Italy are given in the present paper. They show that the Canadian tuckahoe and the European *P. tuberaster* are undoubtedly the same species. These conclusions confirm the view already held by other mycologists (1, 4, 7, 10).

The senior author has examined a sclerotium or 'blackfellow's bread' of the Australian fungus *Polyporus mylittae* Cooke & Massee. Superficially the sclerotium was similar in appearance to that of *P. tuberaster*. The internal tissues of both are made up of dark, gristly portions of coarse, thick-walled hyphae interspersed with grayish-white portions of hyphae of medium thickness. The sclerotial tissue of the Australian fungus was smoother, and, on the whole, lighter in color; but embedded particles of sand and soil, invariably present in the Canadian fungus, were lacking. The two fungi are definitely distinct species.

Interfertility Studies

The data concerning the material used for these studies are as follows:

F 984 – Two small pieces from a sclerotium collected at Grande Prairie, Alta., by J. A. Watt were sent to H. T. Güssow, formerly Dominion Botanist, on Dec. 5, 1928, and cultured by Irene Mounce on Dec. 12, 1928. The fungus fruited in a culture of poplar twigs on malt agar and monospore mycelia were isolated on Feb. 2, 1929, by Mounce (8). A fruit body was again obtained when the culture was transferred by the junior author to an apple pomace and birch sawdust medium* on May 7, 1937, and another series of single spore cultures isolated and given the number F 984A.

F 2345 – Part of a sclerotium found in a field at Raeburn, Man., by David Taylor was sent to the Division of Botany and Plant Pathology, Ottawa, on June 24, 1932. It fruited in culture on apple pomace and birch sawdust, and a series of monospore cultures was isolated on March 17, 1937. Another fruit body was obtained in a similar way on March 29, 1939, and a second series of monospore cultures isolated and given the number F 2345A.

F 2349 – A sclerotium found in a field at Raeburn, Man., by David Taylor on June 11, 1932, was sent to Ottawa by G. R. Bisby and cultured on June 18,

* *Two parts apple pomace to one part birch sawdust by weight plus two-thirds distilled water, sterilized at 15 lb. steam pressure for 30 min.*

1932, by I. Mounce. This specimen was forwarded to the late L. O. Overholts for his herbarium. On April 15, 1939, a Petri dish culture of the fungus on malt agar fruited and shed spores, and a series of single spore cultures was isolated.

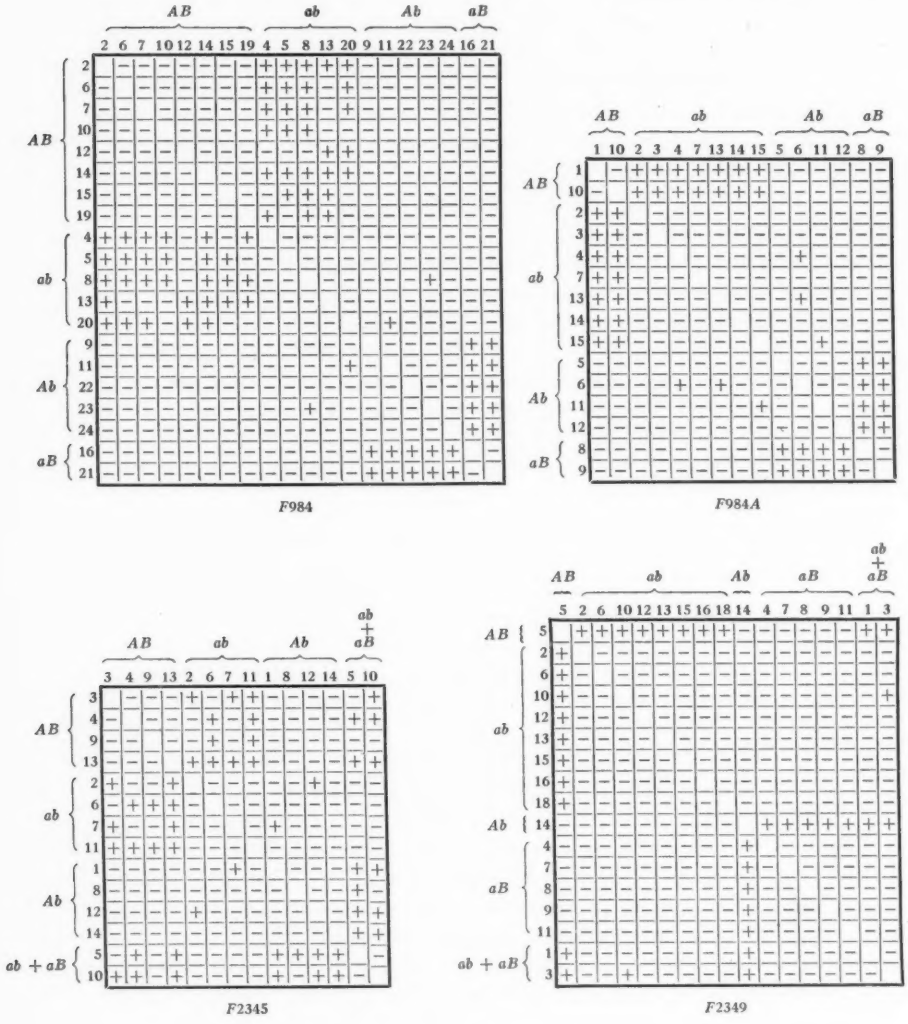

FIG. 10*a*.

FIGS. 10*a* and 10*b*. Tables showing the results of pairing, in all possible combinations, series of monospore mycelia from single sporophores of the Canadian *Polyporus tuberaster* derived from four different sources.

S 147 – A fresh sclerotium collected at Goodsoil, Sask., by Miss Antoinette Schneider, was received on May 29, 1947, and buried 2 in. deep in a poplar grove. It fruited in July and a series of monospore cultures was isolated on July 11. Four monospore cultures representing each mycelial type were later forwarded to the Centraalbureau voor Schimmelcultures, Baarn, Holland.

F 7997 – In the fall of 1937, a sclerotium was collected by G. Goidanich in a forest of beech trees on Mount Termino at Avellino, Italy, and cultured by him at that time. He sent a transfer of the culture to Ottawa in November

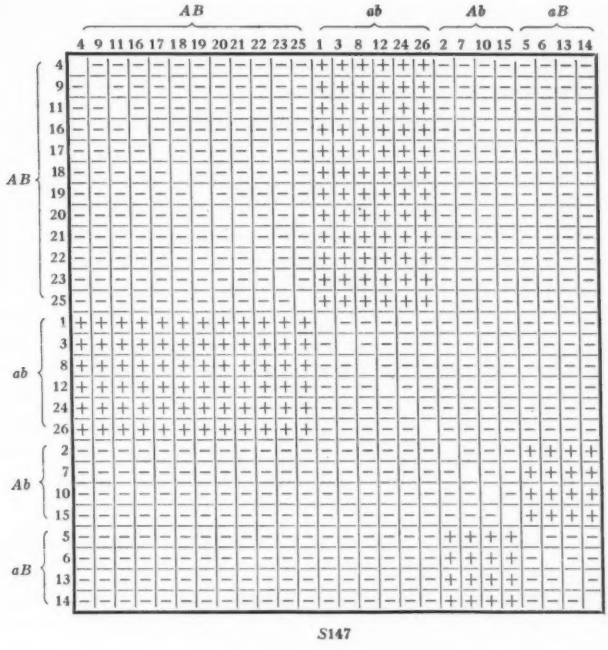

FIG. 10*b*.

of that same year. This culture fruited when placed on an apple pomace and birch sawdust medium, and a series of monospore cultures was isolated on March 27, 1939.

The single spore cultures used in these studies were isolated by obtaining sparsely seeded spore deposits on poured agar plates or by spreading spores, from a deposit on a sterile glass slide, in water over the surface of malt agar or lactose gelatin plates and then cutting out, under the microscope, single germinating spores and transferring them to tubes of malt agar. After the cultures had grown, they were checked microscopically for the absence of clamp connections to ensure that each was from a single spore: multiple

spore cultures of the fungus possess clamp connections but single spore cultures do not. The pairings at Ottawa were made on malt agar in culture tubes and those at Saskatoon, on potato dextrose agar in Petri plates, by placing inocula from two single spore cultures about one centimeter apart on the surface of the culture medium. When the monospore or haploid mycelia had grown together for several days, part of the mycelium lying between the inocula was excised and examined under the microscope for clamp connections. The presence of clamp connections indicated that a diploid mycelium had been formed and that the two haploid mycelia were interfertile; their absence showed that the two mycelia had remained haploid and were intersterile. Many of the paired cultures that, in the first tests, failed to show clamp connections where they were to be expected in the series of pairings, were paired a second time.

In 1930, Mounce (9) reported the results of pairings of monospore mycelia from a fruit body obtained from culture No. F 984. She found clamp connections in a number of the pairings, showing that the species is heterothallic, but repeated trials failed to show definitely whether it is bipolar or tetrapolar. Since that time, other series of pairings of single spore cultures from fruit bodies of the Canadian fungus have been made by Dr. Mounce and by the present authors. Dr. Mounce made another set of pairings with the monospore mycelia of No. F 984. The senior author paired a series of 26 single spore cultures from a sporophore grown from a sclerotium at Saskatoon. The junior author continued the work of Dr. Mounce and paired haploid mycelia isolated from a second fruit body, No. F 984A, originating from the culture used by Dr. Mounce, and also from sporophores from two other sources in Western Canada, Nos. F 2345 and F 2349. The results of these pairings are shown in Fig. 10, where the plus (+) sign indicates the presence of clamp connections, the minus (−) sign their absence.

In all five series of pairings with the Canadian fungus from four different sources, it was found that the haploid mycelia formed four groups according to their pairing reactions, thus showing that the fungus is heterothallic and has the tetrapolar type of interfertility (Fig. 10). Interfertility in tetrapolar species is determined by two pairs of linked factors. In these forms, the diploid nucleus in the basidium has the constitution *AaBb* and gives rise after the reduction division to four types of basidiospores, *AB*, *Ab*, *aB*, and *ab*. In the tables of pairings in Fig. 10, the four interfertility groups present in each series of pairings are assigned these factors. In the series of pairings for No. F 2345, two cultures, Nos. 5 and 10, have paired with the mycelia of two groups instead of only one, as was expected. What had probably occurred with each of these cultures was that two spores had been isolated and that the culture comprised two incompatible haploid mycelia. The constitution of the two mycelia forming the culture may be determined by the fact that the culture paired with isolates of the *AB* and *Ab* groups and would, therefore, possess the opposite pairs of factors, *ab* and *aB*. Similarly, in the series of pairings for No. F 2349, two cultures, Nos. 1 and 3, also appear to have

comprised two incompatible haploid mycelia, *ab* and *aB*. It may be noted that in four of the tables (Fig. 10), those for cultures Nos. F 984, F 984A, F 2345, and F 2349, a few pairings between mycelia that have one factor in common, so-called 'illegitimate' pairings, are recorded. All the illegitimate pairings that have occurred in each of these series have been between mycelia of only two of the four interfertility groups present. Similar findings have been reported by Vandendries (13), Brunswik (3), and Quintanilha (11) in other species. The age of the mycelia when paired was from one to five months.

In addition to the pairings of single spore isolates from Canadian sources, a similar series of pairings of monospore mycelia in all possible combinations from a sporophore arising from the culture received from Italy, No. F 7997, showed that the European *Polyporus tuberaster*, like the Canadian fungus, is heterothallic and has the tetrapolar type of interfertility (Fig. 11). Here, as with three of the Canadian fruit bodies, an illegitimate pairing has occurred.

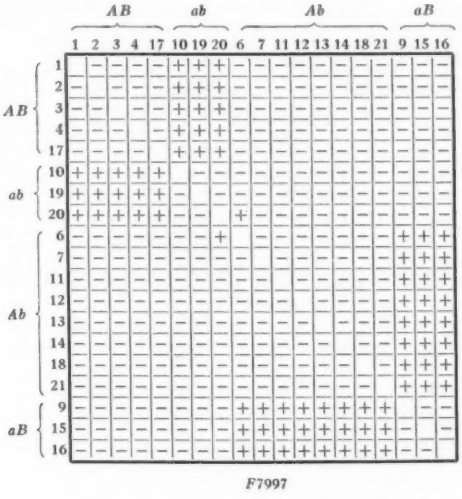

FIG. 11. Table showing the results of pairing, in all possible combinations, a series of monospore mycelia from a single sporophore of the European *Polyporus tuberaster*.

It is known that, in the heterothallic basidiomycetes, when monospore, or haploid, mycelia from sporophores of the same species obtained from different localities are paired together, they are mutually interfertile, but that haploid mycelia from sporophores of different species are intersterile. Conversely, therefore, if haploid mycelia from sporophores obtained from different sources are interfertile, it may be concluded that they are of the same species. In order to prove whether or not the fungus producing the sclerotia found in

Western Canada is the same species as the European *P. tuberaster*, series of pairings of monospore mycelia were made between them. The results of these pairings are given in Fig. 12. One series was made between monospore

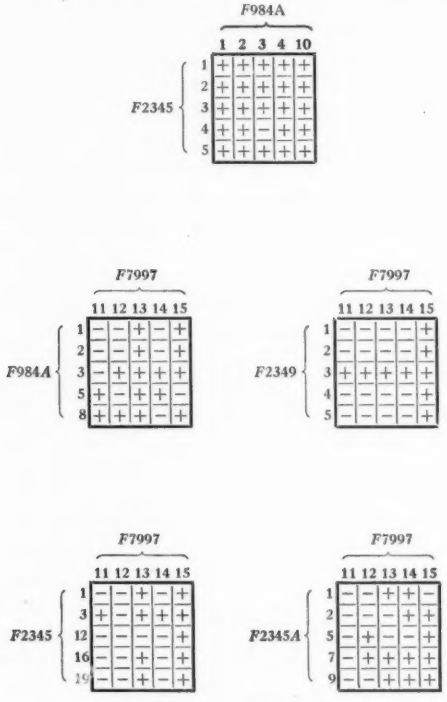

FIG. 12. Tables showing the results of pairing, in all possible combinations, monospore mycelia from single sporophores of the Canadian with the European *Polyporus tuberaster*.

mycelia of two of the Canadian collections, Nos. F 984A and F 2345: with the exception of one pairing, they showed complete interfertility. Four series of pairings were made between single spore cultures of three of the Canadian collections, Nos. F 984A, F 2345 and F 2345A, and F 2349, and the collection from Italy, No. F 7997, known to be *P. tuberaster*. Many of the haploid mycelia in these four series of pairings, though not all, were interfertile. This interfertility between the Canadian fungus and *Polyporus tuberaster* of Europe shows that they are the same species.

Preliminary cultural studies of the monospore mycelia from the Saskatoon fruit body (S 147) revealed that the four haploid types differed from one another in colony characters and rates of growth. Radial growth increments

on malt extract and potato dextrose agars averaged 1 to 2 mm. in 24 hr. at 24° C. The *AB* and *ab* types grew faster and were more consistent in their rates of growth than the *Ab* and *aB* types.

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Written by the author of Field trials: Their Lay-out and Statistical Analysis, published in 1940 and since reprinted; the present bulletin carries the subject into the domain of those topics which are commonly referred to as comprising the "Analysis of Covariance". The object is to assist the investigator still further in reducing his experimental errors by pointing out how observations made on variates other than those in which he is specifically interested may be used by means of a special arithmetical technique, in the statistical analysis of his results.

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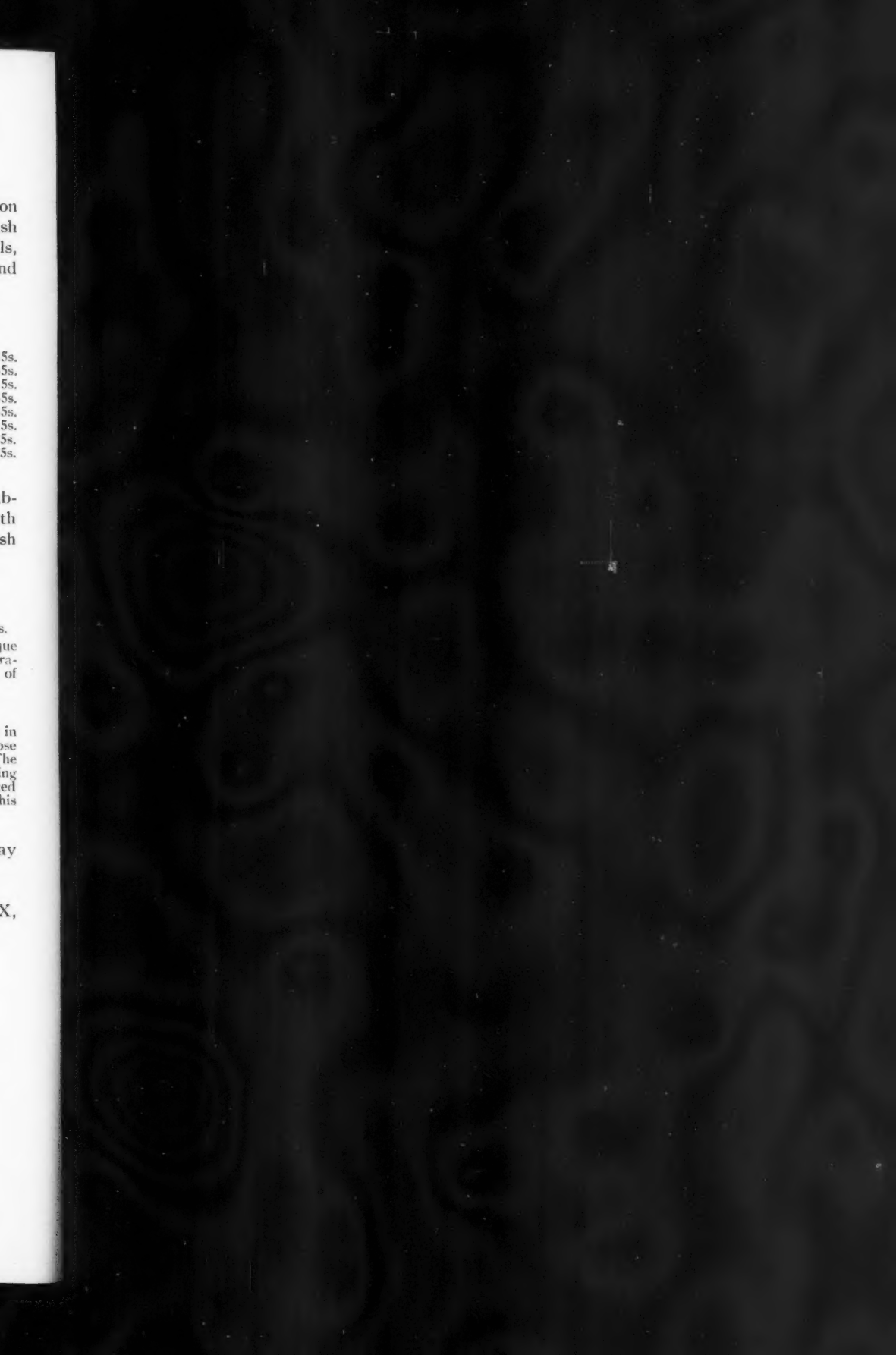

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